Role of gliotoxin in the symbiotic and pathogenic interactions of *Trichoderma virens*

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Using a gene disruption strategy, we generated mutants in the *gliP* locus of the plant-beneficial fungus *Trichoderma virens* that were no longer capable of producing gliotoxin. Phenotypic assays demonstrated that the *gliP*-disrupted mutants grew faster, were more sensitive to oxidative stress and exhibited a sparse colony edge compared with the WT strain. In a plate confrontation assay, the mutants deficient in gliotoxin production were ineffective as mycoparasites against the oomycete, *Pythium ultimum*, and the necrotrophic fungal pathogen, *Sclerotinia sclerotiorum*, but retained mycoparasitic ability against *Rhizoctonia solani*. Biocontrol assays in soil showed that the mutants were incapable of protecting cotton seedlings from attack by *P. ultimum*, against which the WT strain was highly effective. The mutants, however, were as effective as the WT strain in protecting cotton seedlings against *R. solani*. Loss of gliotoxin production also resulted in a reduced ability of the mutants to attack the sclerotia of *S. sclerotiorum* compared with the WT. The addition of exogenous gliotoxin to the sclerotia colonized by the mutants partially restored their degradative abilities. Interestingly, as in *Aspergillus fumigatus*, an opportunistic human pathogen, gliotoxin was found to be involved in pathogenicity of *T. virens* against larvae of the wax moth, *Galleria mellonella*. The loss of gliotoxin production in *T. virens* was restored by complementation with the *gliP* gene from *A. fumigatus*. We have, thus, demonstrated that the putative *gliP* cluster of *T. virens* is responsible for the biosynthesis of gliotoxin, and gliotoxin is involved in mycoparasitism and biocontrol properties of this plant-beneficial fungus.

INTRODUCTION

Gliotoxin is an intriguing natural product of some filamentous fungi, notably the human pathogen *Aspergillus fumigatus* and the plant disease biocontrol agent *Trichoderma* (*Hypocrea*) *virens*. Similar to other ETP (epipolythiodioxopiperazine) compounds, the reactivity of gliotoxin originates from the intact disulphide bridge within the molecule that reacts with thiol groups on proteins, resulting in varied detrimental effects including apoptosis, inhibition of the catalytic activities of the proteasome and angiogenesis (Scharf et al., 2012b). Strong antimicrobial and cytotoxic activity encouraged early attempts to develop this compound as an antibiotic or as a chemotherapeutic agent (Waring & Beaver, 1996). Initially described as a ‘lethal principle’, gliotoxin was first discovered in *T. virens* (misidentified as *Trichoderma lignorum/Gliocladium fimbriatum/Trichoderma viride*) and shown to inhibit growth of the plant pathogen *Rhizoctonia solani* (Weindling, 1934). The active compound was subsequently purified, the structure elucidated and mechanisms of the antimicrobial properties were studied in detail by several groups (Weindling, 1934, 1941; Weindling & Emerson, 1936, Johnson et al., 1943; Brian, 1944; Dutcher et al., 1944; Brian & Hemming, 1945; Wright, 1952; Jones & Hancock, 1988). Despite the discovery of gliotoxin in *A. fumigatus* as early as 1944 (Gister & Williams, 1944; Menzel et al., 1944), the interest in gliotoxin research continued to focus on its relevance in the suppression of plant pathogens (Howell, 2003). The compound was detected in soil and the

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**Abbreviations:** ETP, epipolythiodioxopiperazine; NRPS, non-ribosomal peptide synthetase; PLSD, protected least significant difference; ROS, reactive oxygen species.

Four supplementary figures and one supplementary table are available with the online Supplementary Material.
rhizosphere after application of *T. virens* as a biocontrol agent (Wright, 1952; Lumsden et al., 1992). Using pulse labelling, Willhite & Straney (1996) examined the course of gliotoxin biosynthesis in *T. virens*. The production was demonstrated to commence after 32 h incubation in liquid medium, increase sharply for the next 6 h and then abruptly decline with the last detected labelling occurring at 48 h, representing a duration of de novo biosynthesis of about 16 h. In a significant development for biocontrol of plant pathogens, Howell et al. (1993) found that not all isolates of *T. virens* produce gliotoxin. A proposal reflecting the difference in biosynthesis was presented to designate isolates in one of two groups, consisting of gliotoxin producers, ‘Q’ groups, and non-producers, ‘P’ groups (Howell et al., 1993; Howell & Puckhaber, 2005).

Several studies attempted to establish the role of gliotoxin in the biological control of plant pathogens by generating non-producing mutants through UV mutagenesis (Willhite et al., 1994, Howell & Stipanovic, 1995). In one study, the non-producing mutants displayed significantly less protection of zinnia seedlings against *Pythium ultimum* than the WT strain (Willhite et al., 1994). However, a second study failed to demonstrate a difference between non-producing mutants and the WT parent strain in protecting cotton seedling from disease incited by *R. solani* (Howell & Stipanovic, 1995). The results of these two studies illustrate the complexity of analysing mutants generated by chemical mutagenesis and replication of secondary metabolite production in different strains of the same biocontrol agent. As the application of conventional mutagenesis has the disadvantage of the accumulation of non-target mutations, the interpretation of the different results from these two studies may be due to genetic factors other than mutations in the targeted gene.

Interest in understanding the molecular basis of biosynthesis was renewed once gliotoxin was implicated as a virulence factor in the pathogenesis of the human pathogen *A. fumigatus* (Scharf et al., 2012b). The publication of the genome sequence of this opportunistic human pathogen and subsequent identification of the putative gene cluster (Gardiner & Howlett, 2005; Nierman et al., 2005) enabled the selective inactivation of the core enzyme GliP [a non-ribosomal peptide synthetase (NRPS)] and established the genetic understanding of the first step in gliotoxin biosynthesis (Balibar & Walsh, 2006; Cramer et al., 2006; Kupfahl et al., 2006; Sugui et al., 2007). GliP catalyses the formation of a dipeptide (a fusion of L-phenylalanine and L-serine) followed by cyclization to yield the diketopiperazine (DKP) scaffold. The gene cluster comprises, in addition to *gliP*, 12 other genes that are co-regulated during gliotoxin biosynthesis, with expression regulated by the velvet complex proteins (Perrin et al., 2007; Sugui et al., 2007; Schrettl et al., 2010; Dhingra et al., 2012) and components of the MAPK signalling pathways (Jain et al., 2011). The DKP scaffold is C-hydroxylated by GliC, a putative cytochrome P450 monooxygenase that, after elimination of water, gives rise to imine intermediates that are attacked by the nucleophilic cysteine thiolate residues of two glutathione molecules catalysed by GliG, a glutathione S-transferase (Davis et al., 2011; Scharf et al., 2011; Chang et al., 2013). GliI, a carbon–sulfur (C-S) lyase, catalyses the dual C-S cleavage to yield the epidiethyl moiety of gliotoxin (Scharf et al., 2012a). The disulfide forming oxidoreductase GliT oxidizes this intermediate to yield gliotoxin, which has an intra-molecular disulfide bridge. GliT also mediates self-resistance to gliotoxin in *A. fumigatus* by maintaining the compound with a sulfur-bridge, thus avoiding generation of reactive oxygen species (ROS) and protein conjugates (Scharf et al., 2010, Schrettl et al., 2010). GliZ is a transcriptional regulator of the gliotoxin cluster genes, and the deletion of the gene encoding this protein eliminates gliotoxin biosynthesis and virulence in *A. fumigatus* (Bok et al., 2006). The glutamyltransferase GliK and dipeptidase GliJ are also essential for gliotoxin biosynthesis (Gallagher et al., 2012; Scharf et al., 2013). GliK is induced by exogenous H2O2, and its deletion renders *A. fumigatus* hypersensitive to oxidative stress. Gliotoxin itself is an antioxidant and immunosuppressive metabolite, providing an advantage to *A. fumigatus* during the infection process (Choi et al., 2007; Schrettl et al., 2010; Scharf et al., 2012b).

Contrary to the understanding of the genetic structure and function of gliotoxin in *A. fumigatus*, similar advances have not been demonstrated for this compound that is profusely produced in *T. virens*. The incorrect annotation of a sirodesmin-like gene cluster (*sirP* cluster) as a gliotoxin cluster in *T. virens* hampered early efforts to obtain *gliP* mutants (Patron et al., 2007; C. M. Kenerley, unpublished data). Only recently has the putative gene cluster for gliotoxin biosynthesis been identified following the publication of the genome sequence of *T. virens* (Kubicek et al., 2011; Mukherjee et al., 2012). The putative gliotoxin cluster in *T. virens*, however, is ‘truncated’ comprising only 8 of the 13 genes reported in *A. fumigatus* (Mukherjee et al., 2012). Since the scaffold containing this cluster is small (only these eight genes) and flanked by AT-rich regions, the presence of other members of this cluster elsewhere on the genome is currently unknown. As the seemingly vital genes *gliZ* and *gliT* are absent from this cluster, the *T. virens* cluster appears incomplete. However, the *T. virens* genome does contain two orthologues (EHK22124 and EHK24545) of *gliT* and two orthologues (EHK21730 and ABV48713) of *gliZ* (with more than 50% identity to *A. fumigatus*) located elsewhere in the genome. Unlike *A. fumigatus*, *T. virens* is a beneficial fungus that is found abundantly in soil and the rhizosphere, and offers a wide range of benefits to plants by suppressing pathogenic fungi, promoting photosynthesis and inducing resistance against invading pathogens (Mukherjee et al., 2013). As a first step towards understanding the biosynthesis and biology of gliotoxin in this beneficial fungus, we sought to obtain *gliP* disruption mutants and establish the role of this secondary metabolite during interactions of *T. virens* with plant pathogens and plants.
METHODS

Cultivation of fungi and plants. Two strains of T. virens, Gv29-8 the WT strain and an arginine auxotrophic strain (Tv10.4), and the plant pathogens, P. ultimum, R. solani and Sclerotinia sclerotiorum were used in this study. The fungal strains were routinely cultivated on potato dextrose agar (PDA), and the oomycete on corn meal agar modified with rifampicin. Transformants of T. virens were selected and maintained on PDA modified with hygromycin B (250 and 100 mg 1⁻¹, respectively; PDAH) or Vogel's minimal medium supplemented with 1.5% sucrose (VMS).

Bioinformatics. The sequences of gliP/sirP orthologues from various fungi were downloaded from the National Center for Biotechnology Information site or from their respective genome sites (http://genomewiki.jgi.doe.gov/programs/fungi/index.jsp). The domains of gliP/sirP were identified on the Pfam server (http://pfam.xfam.org/search) and the evolutionary analyses were conducted in MEGA5. Identification of the L-Phe and L-Ser binding domains was performed by alignment using CLUSTAL W software (Stachelhaus et al., 1999; Balibar & Walsh, 2006; Kalb et al., 2013). NRPS predictor 2 was used to identify the signature sequences of the adenylation domains (Röttig et al., 2011).

Construction of gene (gliP) deletion and complementation vectors. The gene deletion cassette used with the WT was constructed by the double joint PCR method (Kuwayama et al., 2002). The left and right flanks were amplified by PCR using the primer pairs GliPupF/GliPupR and GliPDwnF/GliPDwnR (Table S1, available in the online Supplementary Material), respectively. A 1430 bp fragment consisting of the trpC promoter and hygB gene from pCSN43 (Fungal Genetics Stock Center) was amplified with primer pair HygF/HygR. The three fragments were fused by double-joint PCR and the final construct was amplified with the primer pair GliPnestF/GliPnestR. A second gene deletion vector was constructed for use with strain Tv10.4, an auxotrophic strain deficient in the production of arginine. The vector was constructed by cloning a 3 kb fragment of gliP into the plMB4 plasmid containing the arg2 gene (Bae & Kenerley, 1998). The gliP fragment was amplified using the gliP forward primer gliPFWD (5'-GGTCTGGTTCGCGGTGAA-3') and the reverse primer gliPRV2 (5'-CGGGATCCACGCCTTCTTGCCACATGT-3'). Preparation of protoplast- and PEG-mediated transformation of WT with selection for hygromycin resistance or Tv10.4 for arginine prototrophy was performed as previously described (Bae & Kenerley, 1998). Stable prototrophic transformants with hygromycin resistance were selected by consecutive transfer of single colonies to PDAH, PDAH, PDA, and PDAH. A similar serial transfer was conducted to obtain stable transformants for arginine prototrophy (VMS, VMS, PDA, and VMS). The deletion of part of the gliP gene in stable transformants was confirmed by Southern and Northern hybridization (Sambrook et al., 1989).

A mutant (ΔgliP44-4) deficient in the production of gliotoxin in the Tv10.4 background was then complemented with the full-length 7.6 kb GliP gene from A. fumigatus as well as 562 bp upstream and 603 bp downstream regions of the gene (Sugui et al., 2007). Complementation was performed by a co-transformation strategy with a plasmid (pCSN43) containing the hygB gene using hygromycin B as a selectable marker. The absence/presence of the gliP gene was confirmed by PCR (forward primer ΔgliP 5'-ATGCTGGTGACC-TTGCTCAT-3', reverse primer ΔgliP 5'-GCCTGATCCGACAG-GCAGAGA-3'). Production of gliotoxin was confirmed using TLC.

RNA extraction and Northern blotting assays. Total RNA from fungal tissue was prepared using TRIzol reagent (Gibco-BRL). RNA integrity was confirmed after electrophoresis in agarose gels. Samples of RNA were blotted on Hybond-N⁺ membranes (Amersham Biosciences) after electrophoresis according to the manufacturer’s suggestions. The probes were PCR-amplified fragments from fungal genomic DNA. The fragments amplified corresponded to exons of each gene using the primers listed in Table S1 and the correct amplification product was confirmed by sequencing. The purified DNA samples were [³²P] labelled and used for membrane hybridization.

HPLC and TLC analysis. WT and mutant strains were grown in Weindling’s medium (Weindling, 1941) for 4 days, filtered and 10 ml culture filtrate extracted with 20 ml ethylacetate. Samples were air-dried and resuspended in 30 μl methanol. Pure gliotoxin (Sigma) was used as a standard. HPLC was performed as described by Howell et al. (1993) for the detection of gliotoxin, viridin and viridiol at the USDA-ARS-Southern Plains Agricultural Research Center, College Station, TX, USA. Gliotoxin production by the complemented strains was confirmed by TLC. Culture filtrate (10 ml) of strains grown in Weindling’s medium was extracted with ethylacetate, dried under constant air flow and the residue suspended in 20 μl methanol. Samples (10 μl) were loaded onto silica TLC plates and processed using 70:29:1 chloroform : acetone : formic acid running buffer. Plates were visualized under UV light.

Phenotypic analysis. Radial growth of three mutants in the WT background, WT and a strain with an ectopic copy of the deletion vector was determined by placing a 0.5 cm-diameter agar plug from the edge of an actively growing colony for each strain in the centre of a PDA plate. Colony diameter was assessed every 24 h over a 4-day period. There were three biological replications for each strain per experiment with three independent experiments performed. The same plates were used to assess the morphology and branching of hyphae of each strain at the edge of each developing colony using an Olympus BX60 compound microscope. Q Imaging go-21 camera and QCapture software. For assessing the sensitivity of the strains to oxidative stress, spore suspensions (10⁷ spores ml⁻¹) of each strain were prepared from 10-day-old cultures. A 3 μl drop was placed in the centre of a plate containing PDA supplemented with 1 or 10 mM H₂O₂. For controls, the strains were inoculated on plates without the addition of H₂O₂. The plates were incubated at 27 °C in plastic boxes with saturated paper towels to maintain humidity. The radial growth was recorded every 24 h in three independent experiments (biological replications) that included three plates for each strain.

Confrontation and hyphal coiling. Ability of the ΔgliP mutants to overgrow and lyse the colonies of the pathogens P. ultimum, R. solani and S. sclerotiorum was assessed by placing pairs of the fungi approximately 60 mm apart opposite each other on PDA plates. The plates were observed daily over a 7-day period for overgrowth of Trichoderma on the pathogen colonies. To observe hyphal coiling by strains of T. virens, 1 ml VMS was pipetted onto glass microscope slides. The slides were then inoculated with a plug of strains WT, ΔgliP6, ΔgliP13 or ΔgliP14. After incubating for 12 h at 27 °C, the slides were then inoculated at the opposite end of the slide with a plug of R. solani. After 24 h co-incubation, microscopic observations of the interaction zone were performed.

Interactions with sclerotia. Ability of WT and ΔgliP mutants to attack the sclerotia of S. sclerotiorum was assessed in 24-well plates containing 1 ml PDA per well. Each well was seeded with a conidial suspension (10 μl of 10⁷ conidia ml⁻¹) of the appropriate strain, and plates were sealed with Parafilm and incubated for 7 days at 25 °C. Sclerotia were harvested from 7-day-old cultures of S. sclerotiorum, and one sclerotium was placed in each pre-inoculated well. Prior to placing the sclerotia into the wells, each well was visually assessed to determine that the entire well was colonized with hyphae of the appropriate strain of T. virens. The plates were resealed and incubated for an additional 11 days. In a second trial, the sclerotia were dipped in gliotoxin (20 mg ml⁻¹ in methanol) or methanol (control) for approximately 30 s before placing into wells pre-inoculated with P. ultimum.
RESULTS

Deletion of gliP abolishes gliotoxin production in T. virens

Using double-cross-over homologous recombination, we obtained mutants in the T. virens gliP gene with hygB gene as the selectable marker. The gene disruption was confirmed by Southern blotting analysis (Fig. 1a, b). Null expression of the gene was determined by Northern blotting (Fig. 1c). The mutants did not produce gliotoxin as determined by HPLC analysis of the culture filtrate (Fig. 1d). However, the mutants did produce the fungistatic compound viridin and the mycoherbicide viridiol that are characteristic of the WT strain (Fig. 1d) (Jones & Hancock, 1987). Overall, these results confirmed the inactivation of gliP in T. virens and correlated the functional synthesis of the gene product with gliotoxin accumulation.

We also studied the effect of gliP disruption on the expression of other genes of the cluster (gliC, gliF, gliG, gliI, gliK, gliM and gliN). Northern blot analysis indicated that transcription of all these genes are gliP (or possibly gliotoxin) dependent, though their expression was not completely repressed in the gliP mutants (Fig. 2).

Phenotypic analysis

Compared with WT, the mutants showed significantly enhanced radial growth after 48 h incubation on PDA plates (Fig. 3a, b), VMS, malt extract agar or water agar (data not shown), thus, providing evidence that gliotoxin biosynthesis has negative effects on vegetative growth in the fungus. An examination of the mycelia at the edge of the advancing colony of the mutants on PDA illustrated morphology that was more dispersed, less dense and less branched as compared with the WT (Fig. 3c). Complementation of a gliP mutant with the A. fumigatus gliP gene (Sugui et al., 2007) restored the production of gliotoxin (Fig. S1).

Gliotoxin mutants are hypersensitive to oxidative stress

The sensitivity of the mutants to oxidative stress was assessed by incorporating H2O2 in the medium and measuring colony growth at regular intervals. As previously observed, the mutant strains exhibited significantly greater colony growth than the WT strain on PDA without H2O2 (Fig. 4). When the strains were compared in the presence of 1 mM H2O2, the ΔgliP strains demonstrated considerable sensitivity to the oxidative stress. As illustrated in Fig. 4, the colony area of the ΔgliP strains was reduced by approximately 50% compared with their growth on PDA. At this concentration of H2O2, there was no significant difference in growth among the strains. The importance of gliotoxin for oxidative-stress tolerance was further confirmed when comparing the effect of 10 mM H2O2 on colony growth. In this case, a 50% reduction in growth rate was observed in the WT strain compared with control plates (no H2O2 added). In contrast, the mutant strains were severely affected by the addition of 10 mM H2O2, displaying up to 90% reduction in growth compared with Gv29-8 under the same condition. The three mutants were similar, but Gv29-8 had significantly greater growth (Fig. 4).
Gliotoxin is involved in antagonism and disease biocontrol, but not in internal colonization of roots

_T. virens_ is a mycoparasite and capable of overgrowing colonies of some plant pathogens in dual culture. Strains are also effective agents for degradation of the resting structures known as sclerotia (Mukherjee _et al._, 1995). As expected, WT continued to grow over the colonies of _P. ultimum_ and _S. sclerotiorum_ in the confrontation assay (Fig. 5). Deletion of gliP, however, adversely affected the antagonism of _T. virens_. Instead, the pathogens overgrew the colony of _T. virens_ (Fig. 5), indicating attenuation of mycoparasitism in the absence of gliotoxin production, despite faster growth rate of the mutants in pure culture. All strains were capable of overgrowing colonies of _R. solani_ (Fig. 5). Also, both the mutants and the WT strain were capable of coiling around the hyphae of _R. solani_ (data not shown). The absence of gliotoxin production adversely affected not only hyphal parasitism, but also the ability to degrade the sclerotia of _S. sclerotiorum_. The WT strain was found to alter the physical integrity of the wall of the sclerotia more significantly than either of the mutants (Fig. 6a). Although, the mutants were significantly reduced 

\[ P<0.01 \]

in their ability to attack the sclerotia, an

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**Fig. 1.** Gliotoxin production is abolished in _T. virens_ due to deletion of part of the _gliP_ gene. (a) The scheme depicts the strategy for gene deletion through homologous recombination. After recombination, the hygromycin cassette is inserted into the genomic region disrupting the GliP-encoding region. (b) Southern hybridization of genomic DNA extracted from the WT and ΔgliP mutant strains using the probe indicated in panel (a). Arrows on the right indicate the expected band size for both native and deletion events. The minor non-specific bands appear to be due to non-selective hybridization with other NRPSs. (c) Comparison of the accumulation of mRNA for _gliP_ in the WT and mutant strains. Fungal mycelium was incubated in Weindling’s medium for 96 h and total RNA extracted for Northern hybridization. Fifteen micrograms total RNA were electrophoresed in agarose gels and blotted on nitrocellulose membranes. The probe used for the hybridization was PCR-amplified from genomic DNA using the primers GliPF/GliPR and radioactively labelled with \([^{32}P]dCTP\). (d) HPLC detection of gliotoxin in culture filtrates from Gv29-8 and ΔgliP6.
planted with just chlamydospores of the mutants was similar to seeds with no treatment. A comparison among the two treatments indicated that the WT offered significant increase in seedling survival compared with the mutants. The mutants had impaired biocontrol ability in the protection of cotton against seedling susceptibility by WT (Fig. 6b). The mutants also showed no level of control, as survival of seedlings coated with chlamydospores to cotton seeds resulted in a lower conidial concentration (10^5 conidia ml⁻¹) compared with what is known in the genetic system for biosynthesis in R. solani.

Gliotoxin is involved in entomopathogenic properties of T. virens

An infection assay of G. mellonella larvae indicated that gliotoxin is indeed involved in the ability of T. virens to kill insects. In our assay conditions, there was lower mortality in the gliotoxin mutants compared with the gliotoxin-producing WT strain at both conidial concentrations injected into the larvae. However, the effect was more pronounced when 5 μl of 10^7 conidia ml⁻¹ were injected compared with a lower conidial concentration (10^5 conidia ml⁻¹) (Fig. 8).

The two adenylation (A) domains of the ETP NRPs have evolved independently

A phylogenetic analysis of the first (A1) and second (A2) adenylation domains of fungal NRPs involved in ETPs (GliP/SirP) biosynthesis indicated that these two domains evolved independently and not by duplication (Fig. S3). Also, the phylogenetic relatedness of both the domains of T. virens to those of A. fumigatus is indicative of a possible horizontal gene transfer. Interestingly, Trichoderma reesei also harbours a putative (partial) gliotoxin cluster even though this fungus does not produce gliotoxin and is not a biocontrol strain. The A1 domains of GliP and SirP are closer to each other (the case with A2 domains is similar) than to their own A2 domains, and it is possible that these A1 domains of SirP and GliP and A2 of SirP and GliP have a common origin. An analysis of the L-Phe-activating adenylation domains indicated that the putative amino acid-activating residues are identical in A. fumigatus and T. virens. The same is true for the L-Ser-activating adenylation domain residues (Stachelhaus et al., 1999) (Fig. S4).

DISCUSSION

Gliotoxin was first discovered in T. virens and studied for its ability to suppress plant pathogens (Weindling, 1934). Even though T. virens has the ability to produce copious amounts of this secondary metabolite, an understanding of the genetic system for biosynthesis in T. virens has lagged compared with what is known in A. fumigatus, the other economically important fungus that produces gliotoxin. Previous studies have demonstrated that a gli cluster resides in T. virens that is similar to the gli cluster in A. fumigatus, members of the gli cluster are co-regulated during interactions with the plant pathogen R. solani, and gliotoxin production is regulated by the velvet complex protein Vel1 (Mukherjee & Kenerley, 2010; Kubicek et al.,...
Our research is, to the best of our knowledge, the first genetic study to demonstrate that the cluster is responsible for biosynthesis of gliotoxin in *T. virens* and represents a significant first step in understanding the biology of gliotoxin biosynthesis in this plant-beneficial fungus.

The role of gliotoxin in biocontrol of soil-borne plant diseases was earlier examined by obtaining non-producing mutants through classical chemical mutagenesis (Howell & Stipanovic, 1995; Wilhite & Straney, 1996). However, each group used a different parent strain to generate their mutants and evaluated the constructed mutants against a different plant pathogen. The application of different strains of *T. virens* has demonstrated a strain effect on the efficacy of disease control against the pathogens *P. ultimum* and *R. solani* (Lumsden & Locke, 1989; Burns & Benson, 2000; Lewis & Lumsden, 2001; Dubey et al., 2011).

Q strains (which produce gliotoxin) were shown to be effective against *R. solani* while P strains (which produce gliovirin, but not gliotoxin) were effective against *P. ultimum*, and not vice versa (Howell et al., 1993). By testing mutants in the same genetic background derived by selected gene disruption of gliP against the pathogens *P. ultimum* or *R. solani*, we sought to resolve the role of gliotoxin against these important soil-borne pathogens. Our results confirm previous studies in that gliotoxin is clearly involved in protection of seedlings against *P. ultimum*, but not *R. solani*. Mutant and WT strains demonstrated similar levels of control against *R. solani*, but the mutants were greatly impaired in their ability to control *P. ultimum*. Our assay did not differentiate whether...
the control was the result of mycoparasitism of pathogen propagules or induction of resistance in the host. We have previously demonstrated that mutants lacking the ability to produce an endochitinase are impaired in their ability to protect cotton against *R. solani* (Baek et al., 1999). By constitutively expressing two glucanases in WT, we also showed an enhanced ability to protect cotton seedlings against the same pathogen (Djonović et al., 2007). Even though glucanases have been demonstrated to be significantly involved in the biocontrol ability of *T. virens* against *P. ultimum* (Djonović et al., 2006, 2007), perhaps the combination of these enzymes and gliotoxin is necessary to prevent infection of seeds and newly formed roots by this soil-borne pathogen. The effect of gliotoxin on *P. ultimum* may be explained by differential interaction with the cell wall of an oomycete, which is in a distinct phylogenetic lineage from filamentous fungi. Gliotoxin and other ETPs such as sirodesmin exhibit phytotoxic properties, which may differentially have a greater effect on oomycetes. In addition, we demonstrated that gliotoxin does play a role in sclerotial degradation, which is useful for strain selection. Sclerotia are resistant to many environmental conditions. Gliotoxin mainly affects metabolic functions and is not known to have a lytic function. Therefore, an assumption would be that gliotoxin is not responsible for the degradation of sclerotia, but rather facilitates the colonization of the resting structure by *T. virens*. The mechanism(s) enabling gliotoxin to exert such an effect remains to be studied. Another interesting feature is that gliotoxin deficiency did not affect the ability of *T. virens* to penetrate and colonize roots internally. Successful root colonization by *T. virens* and other beneficial fungi is a complex interaction involving signalling events between the host and putative symbiont. Colonization requires an ability to recognize and adhere to roots, penetrate the plant and initially withstand any toxic metabolites produced by the plant in response to the invasion. *T. virens*

![Fig. 5. Gliotoxin biosynthesis differentially contributes to the antagonistic effect of Gv29-8 against *P. ultimum*, *S. sclerotiorum* and *R. solani*. Dual cultures of strains Gv29-8 and ΔgliP13, and the pathogens *P. ultimum*, *S. sclerotiorum* and *R. solani*, on PDA medium. Plates were incubated at 25 °C for 7 days.](image1)

![Fig. 6. Sclerotia susceptibility assay. (a) Degradation index after 11 days’ incubation in the presence of different strains of *T. virens*; (b) sclerotia were dipped in a gliotoxin solution (20 mg ml⁻¹ in methanol) or in methanol as a control, prior to exposure to *T. virens*. The bars represent the mean value ± SD of three independent experiments; different symbols (*, †, ‡, §) represent significant differences (P<0.01) according to Fisher’s PLSD test.](image2)
and other species grow intercellularly in the root epidermis and cortex, but persistent and continued hyphal growth in this portion of a living root after ingress by *Trichoderma* requires damping or repressing the plant defence responses (Druzhinina *et al.*, 2011). Once hyphae of *T. virens* encounter roots of a seedling of any number of crop hosts, the process of colonization is initiated, secreted effectors are delivered to the surface or the interior of plant cells and the plant responds to the intruder (Harman *et al.*, 2004). Despite the demonstration by Lumsden *et al.* (1992) that gliotoxin is produced in soil and soilless media, the compound does not appear to be necessary in this competitive environment to enhance or control root colonization.

Using a targeted gene strategy, we have shown that gliotoxin negatively affects vegetative growth and alters colony morphology in the producing strain. These phenotypic changes are in contrast to *A. fumigatus* where disrupting the production of gliotoxin did not alter colony growth. However, the role of gliotoxin in oxidative-stress tolerance is similar to that observed in *A. fumigatus* (Scharf *et al.*, 2010; Schrettl *et al.*, 2010).

ETP toxins such as gliotoxin can perform what is known as redox cycling in which the disulfide bond is broken via reduction and auto-oxidized into its disulfide form producing ROS (Gardiner *et al.*, 2005; Scharf *et al.*, 2010). This redox cycling may be important for the toxicity of gliotoxin. As *T. virens* is noted for producing large quantities of gliotoxin (Lumsden *et al.*, 1992), this fungal strain most likely co-evolved a mechanism enabling the cells to cope with or tolerate large concentrations of ROS. Our results indicate a significant difference in oxidative stress tolerance in *T. virens* strains impaired in gliP expression as compared with the WT strain Gv29-8. Even though these results suggest a connection between this ETP compound and the mechanisms involved in detoxification of exogenous H$_2$O$_2$, the precise mechanisms are not fully understood. In *A. fumigatus* it was demonstrated that normal expression of gliK is important for gliotoxin accumulation and in coping with oxidative stress (Gallagher *et al.*, 2012). In *T. virens*, the sensitivity of the gliP-disrupted mutants to oxidative stress may be related to either a co-regulation of gliP and ROS-detoxifying mechanisms or a direct effect of gliotoxin per se on H$_2$O$_2$. Further investigations will be necessary to provide a better understanding of the mechanisms involved in gliotoxin-mediated H$_2$O$_2$ tolerance by *T. virens*.

This study provides the first documented evidence that the putative gliotoxin cluster found in the *T. virens* genome is indeed a gli cluster, and future studies should focus on the role of individual genes in the cluster in gliotoxin biosynthesis in *T. virens*, as well as the regulation of this cluster. Such studies will not only help in the understanding of the gliotoxin-dependent biology of this biocontrol fungus, but also bolster our understanding of gliotoxin biosynthesis in general, having a bearing on *A. fumigatus* biology as well. Moreover, since gliotoxin is regarded as a mycotoxin, better understanding of gliotoxin biosynthesis and its role in *T. virens* would also aid in finding ways of minimizing gliotoxin ‘load’ in the environment. The potential for gliotoxin use in biocontrol applications as a...
formulated product or in transgenic or overexpressing strains needs to be assessed in field trials. The anti-cancer effects of this reactive compound warrant further examination as ETPs and ETP-like alkaloids have exhibited potent anti-cancer roles via induction of apoptosis (Boyer et al., 2013). However, negative effects on non-cancerous cells are a concern. Potentially the use of a gli cluster gene product in conjunction with an ETP such a gliotoxin may find use in emerging cancer treatments such as antibody-drug conjugate therapy. This therapy involves the use of a therapeutic toxin bound to an antibody that is specifically delivered to a cancerous area. Such treatments have already been approved by the US Food and Drug Administration (Teicher & Doroshov, 2012; Flygare et al., 2013). Further studies on gli cluster gene products may reveal the potential for cancer cell-specific treatments. The mining of microbial secondary metabolites has the potential for discovery of compounds that can be implemented for the protection of both field crops and mammalian diseases.

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REFERENCES


Trichoderma virens gliotoxin


