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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 to 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 to 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.

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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; Dutcher *et al.*, 1944; Brian, 1944; Brian & Hemming, 1945; Wright, 1952; Jones & Hancock, 1988). Despite the discovery of gliotoxin in *A. fumigatus* as early as 1944 (Menzel *et al.*, 1944; Glister & Williams, 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

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.

soil and the rhizosphere after application of *T. virens* as a biocontrol agent (Wright, 1952; Lumsden *et al.*, 1992). Using pulse labelling, Wilhite & 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 declining 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 (Wilhite *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 (Wilhite *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 other genetic factors 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 (Nierman *et al.*, 2005; Gardiner & Howlett, 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 (Dhingra *et al.*, 2012; Perrin *et al.*, 2007; Sugui *et al.*, 2007; Schrettl *et al.*, 2010) 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 (Scharf *et al.*, 2011; Davis *et al.*, 2011; Chang *et al.*, 2013). GliI, a carbon–sulfur (C-S) lyase, catalyses the dual C-S cleavage to yield the epidithiol moiety of gliotoxin (Scharf *et al.*, 2012a). The disulphide forming oxidoreductase GliT oxidizes this intermediate to yield gliotoxin, which has an intra-molecular disulphide 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 H_2O_2, and its deletion renders *A. fumigatus* hypersensitive to oxidative stress. Gliotoxin itself is an anti-oxidant and immuno-suppressive 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 l^{-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://genome.jgi.doe.gov/programs/fungi/index.jsf). 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 to 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 pJMB4 plasmid that contains the *arg2* gene (Baek & Kenerley, 1998). The *gliP* fragment was amplified using the *gliP* forward primer *glip*FWD (5′-GGTCTGGTTCGCGGTGAA-3′) and the reverse primer *glip*REV2 (5′-CGCGGATCCACGCCTTCT-GCCACACTG-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 (Baek & 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 (Δ*gliP*44-4) deficient in the production of gliotoxin in the Tv10.4 background was then complemented with the full-length 7.6 kb *AfgliP* 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 AfgliP 5′-ATGCTCGTGACC-TTGCTCAT-3′, reverse primer AfgliPR 5′-CGCCATGCAGCAAC-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 [^{32}P] 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 were 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 assaying the sensitivity of the strains to oxidative stress, spore suspensions (10^7 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 H_2O_2. For controls, the strains were inoculated on plates without the addition of H_2O_2. 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 Δ*gliP* 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 WT, Δ*gliP*6, Δ*gliP*13 or Δ*gliP*14 strains. 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 Δ*gliP* 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 10^7 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 culture 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

conidia of the WT or mutants. Susceptibility of sclerotia to attack by the strains of *T. virens* was determined by simple pressure test with forceps and rated from 1 to 3: 1, complete softening with collapse of the integrity of the sclerotial wall and contents; 2, partial effect with pressure required to indent the sclerotium with the forceps; and 3, no effect with the sclerotial wall intact. The control (no strains of *T. virens*) sclerotia were assayed first to determine the integrity and amount of pressure to apply to the other treatments. The experiment was repeated three times with six replicates, each well being considered a replicate for a total of 18 experimental units per treatment.

Root colonization assay. The ability of WT and the Δ*gliP* mutants to internally colonize maize roots was assessed by the method described earlier (Vargas *et al.*, 2009). Briefly, maize seeds were planted in glass tubes (18 × 150 mm) each containing 10 g non-sterile soil that was infested with an aqueous conidial suspension (10^7 conidia ml^{-1}, 1 ml per tube) of the appropriate strain. The tubes were incubated in a growth chamber at 25 °C with a 16 : 8 h light : dark cycle with constant humidity. Seedlings were harvested after 4 days, and the root surface disinfested with1 % NaClO. Roots were ground in 100 mM sodium phosphate buffer (pH 7) with 20 mM $MgCl_2$ and Silwet L-77 (1 g roots in 5 ml solution) and plated on *T. virens* selective medium (GVSM) minus gliotoxin (Park *et al.*,1992). *Trichoderma* colonies were counted after 3 days. Roots from five seedlings were combined for one replication, each treatment was replicated four times, and the entire experiment repeated.

Biocontrol assay in a growth chamber. The ability of WT and Δ*gliP* strains to protect cotton seeds/seedlings from the pathogens *P. ultimum* or *R. solani* was assessed in non-sterile soil in test tubes (18 × 150 mm) (Djonović *et al.*, 2007). Briefly, cottonseeds were coated with a chlamydospore preparation (Weaver & Kenerley, 2005) of the appropriate strains and sown in soil pre-infested with *P. ultimum* oospores or wheat bran colonized by *R. solani*. The assay tubes were incubated for 7 days in a growth chamber at 27 °C with a light : dark cycle of 14 : 10 h. Seedling survival was enumerated using an index from 1 to 3: 1, dead with extensive lesions or shrivelled roots; 2, discoloured lesions apparent; 3, generally healthy with no signs of seedling damage. There were 10 tubes per replication, with four replications per experiment and the entire experiment was repeated three times.

Mortality of wax moth (*Galleria mellonella*) larvae. Final instar larvae of *G. mellonella* (Vanderhorst Wholesale) were injected with 5 µl conidial suspension (10^5 or 10^7conidia ml^{-1} in PBS) of WT or Δ*gliP* mutant. Larvae were injected via the last left proleg, incubated at 25 °C in the dark (Jackson *et al.*, 2009; Julie *et al.*, 2006) and monitored daily for 3 days to record mortality. There were 12 larvae per treatment with three replications and the entire experiment was performed twice.

Statistical analysis. Statistical analyses were performed for the appropriate experiments using ANOVA and mean separation by Fisher's protected least significant difference (PLSD) test ($P<0.05$ or $P<0.01$) (Statview; SAS Institute).

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 ~~blot~~ analysis (Fig. 1a, b). Null expression of the gene was determined by Northern ~~blot~~ (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). All together, 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*). The Northern blot analysis indicated that transcription of all these genes are *gliP* (or perhaps gliotoxin) dependent, though their expression was not completely repressed in the *gliP* mutants (Fig. 2).

Phenotypic analysis

Compared with WT, the mutants had a significantly enhanced radial growth after 48 h incubation on PDA plates (Fig. 3a, b), VMS, malt extract agar and 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 to 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 H_2O_2 in the medium and measuring the colony growth at regular intervals. As previously observed, the mutant strains exhibited significantly greater colony growth than the WT strain on PDA without H_2O_2 (Fig. 4). When the strains were compared in the presence of 1 mM H_2O_2, the Δ*gliP* strains demonstrated a considerable sensitivity to the oxidative stress. As illustrated in Fig. 4, the colony area of the Δ*gliP* strains was reduced approximately 50 % compared to their growth on PDA. At this concentration of H_2O_2, 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 H_2O_2 on colony growth. In this case, a 50 % reduction of growth rate was observed in the WT strain compared to control plates (no H_2O_2 added). In contrast, the mutant strains were severely affected by the addition of 10 mM H_2O_2, displaying up to a 90 % reduction of growth compared to Gv29-8 in the same condition. The three mutants were similar, but Gv29-8 had significantly greater growth (Fig. 4).

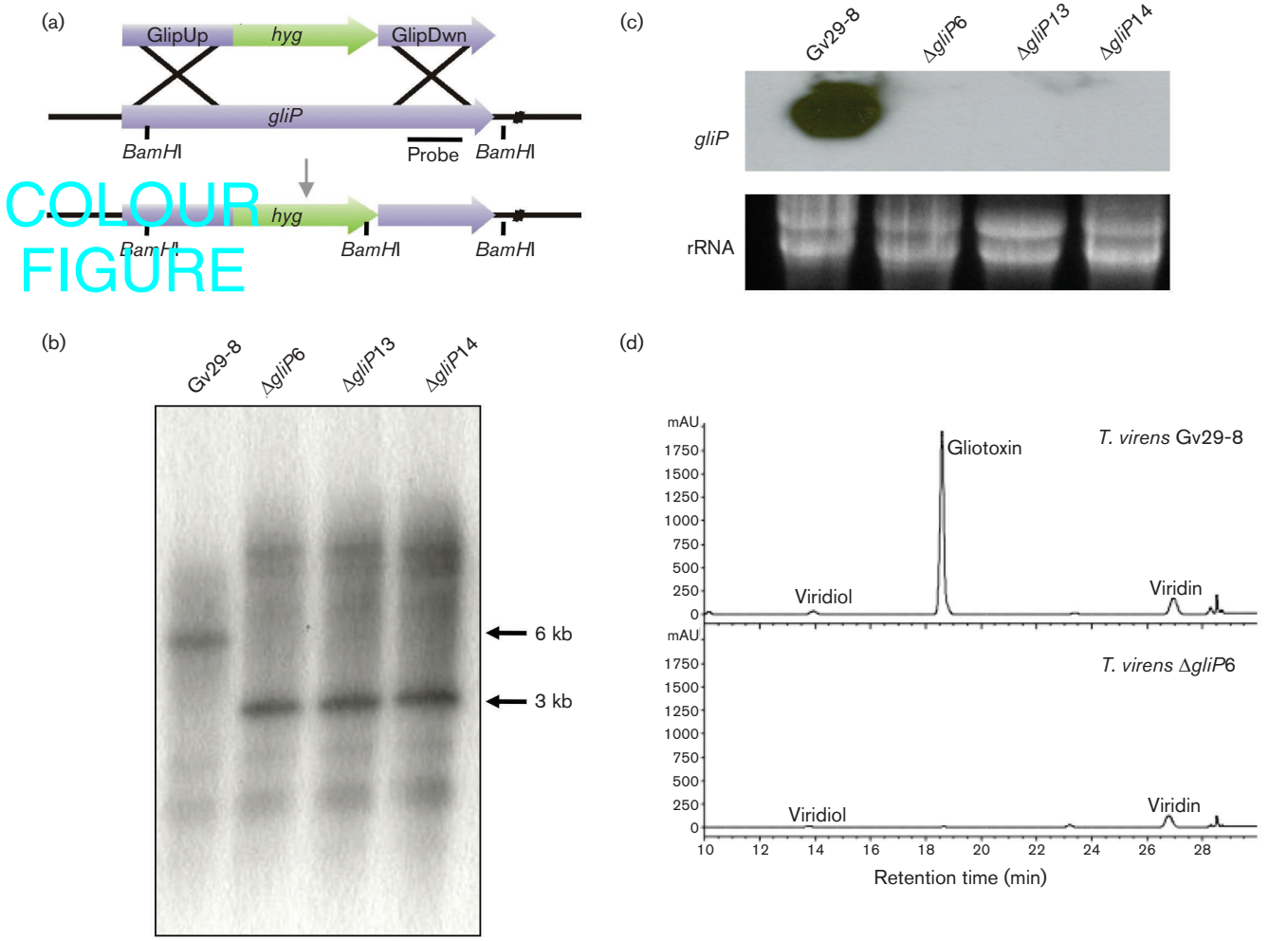

Fig. 1. Gliotoxin production is abolished in *T. virens* due to deletion of part of the *gliP* gene. (a) The scheme depicts the strategy for gene deletion through homologous recombination. After the recombination, the hygromycin cassette is inserted in the genomic region disrupting the GliP-encoding region. (b) Southern hybridization of genomic DNA extracted from the WT and Δ*gliP* mutant strains using the probe indicated in panel (a). Arrows on the right indicate the expected band sizes for both native and deletion events. The minor non-specific bands appear to be due to non-selective hybridization with other NRPS(s). (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 Δ*gliP*6. [9]

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 an attenuation of mycoparasitism in the absence of gliotoxin production, despite faster growth rate of the mutants in pure culture. All of the strains were capable of overgrowing colonies of *R. solani* (Fig. 5). Also, the mutants as well as 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 significantly more than either of the mutants (Fig. 6a). Although, the mutants were significantly reduced ($P<0.01$) in their ability to attack the sclerotia, an

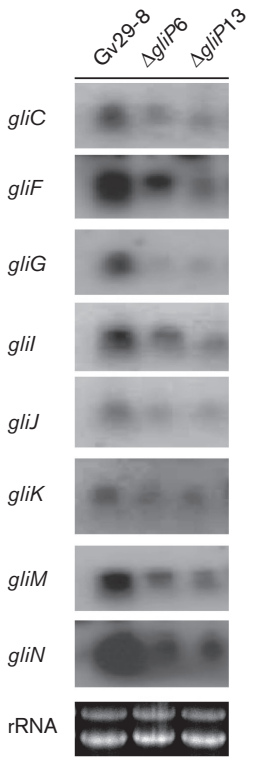

Fig. 2. Expression of genes in the gliotoxin cluster is dependent on *gliP*/gliotoxin. Total RNA samples were prepared from fungal mycelia incubated for 96 h in Weindling's medium. Fifteen micrograms total RNA were electrophoresed in agarose gels and blotted on nitrocellulose membranes. The probe used for the hybridization was PCR amplified from genomic DNA using the primers listed in Table S1 and radioactively labelled with [^{32}P]dCTP.

exogenous application of gliotoxin to the sclerotia prior to incubation with the putative antagonists partially restored this property in the mutants (Fig. 6b). That is, the mean separation test demonstrated a significant difference between mutants with or without gliotoxin, but both were significantly less able to attack sclerotia than WT. The addition of exogenous gliotoxin to sclerotia prior to adding the sclerotia to wells containing WT did not enhance the sclerotia susceptibility by WT (Fig. 6b). The mutants also had impaired biocontrol ability for the protection of cotton seedlings against *P. ultimum* in a growth chamber assay after 7 days incubation (Fig. 7). A comparison among the treatments indicated that the WT offered significant protection to cotton seedlings against the pathogen compared to the ability of the mutants. The mutants showed no level of control as survival of seedlings coated with chlamydospores of the mutants was similar to seeds planted with just *P. ultimum*. In contrast, the mutants were not significantly different from the WT in their ability to protect seedlings against *R. solani*. Application of mutants or WT as chlamydospores to cotton seeds resulted in a significant increase in seedling survival compared to the control (*R. solani* alone treatment). Treatments with WT and mutants resulted in >85 % survival compared to 25 % in treatment with just R. *solani* (Fig. S2). In another standard assay, the mutants were found to retain their ability to invade and internally colonize maize roots suggesting that gliotoxin does not play a significant role in root colonization (data not presented).

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 less mortality in the gliotoxin mutants compared to the gliotoxin-producing WT strain at either conidial concentration injected into the larvae. However, the effect was more pronounced when 5 µl of 10^7 conidia ml^{-1} were injected compared to a lower conidia concentration (10^5 conidia ml^{-1}) (Fig. 8).

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

A phylogenetic analysis of the first (A1) and the second (A2) adenylation domains of fungal NRPSs 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 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 to 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 (Kubicek *et al.*, 2011; Mukherjee *et al.*, 2012;

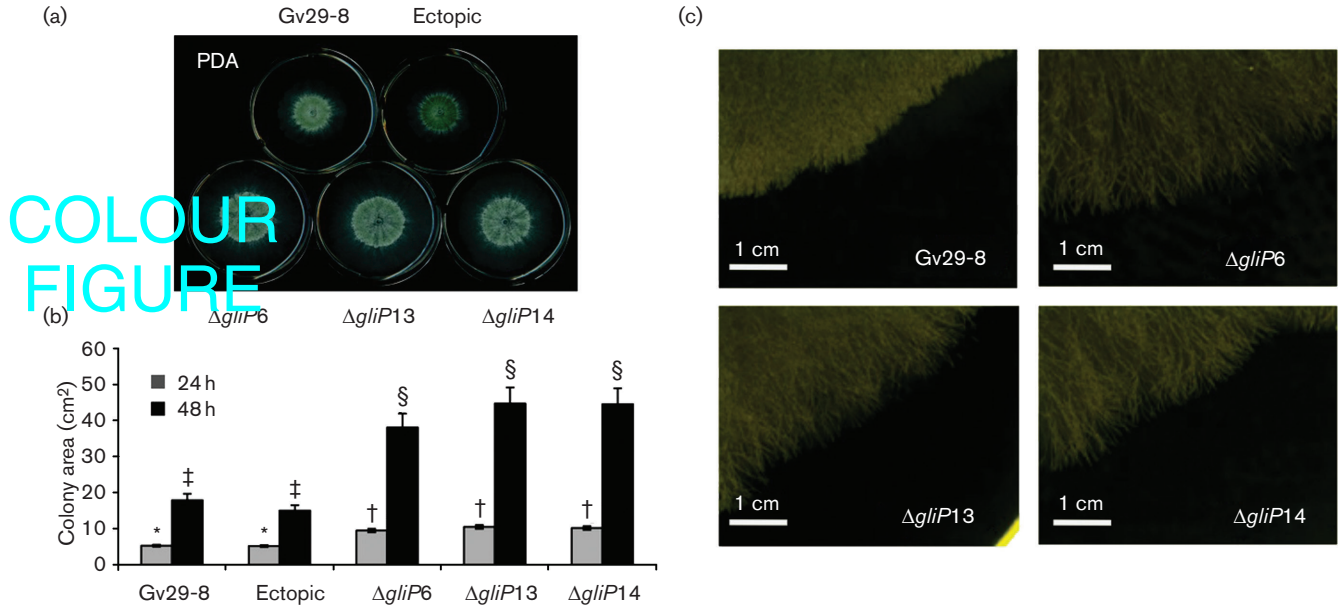

Fig. 3. Effect of *gliP* deletion on *T. virens* development. (a) Colony development on PDA plates. Agar plugs containing actively growing mycelia from strains Gv29-8, Δ*gliP*4, Δ*gliP*13, Δ*gliP*14 and a strain containing an ectopic integration of the replacement cassette were placed in the centre of the plate and photographed 4 days after inoculation. (b) Colony area was determined 24 and 48 h after inoculation in the PDA plate. The quantitative data confirmed the higher growth rate for the mutant strains. The bars depict the mean ± SD of three independent experiments. Different symbols (*, †, ‡, §) indicate significant differences with a $P<0.01$ according to Fisher's PLSD test. (c) The edge of the colony was photographed 4 days after inoculation of a PDA plate. Similar results were observed in three independent experiments.

Mukherjee & Kenerley, 2010; Atanasova *et al.*, 2013). 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.

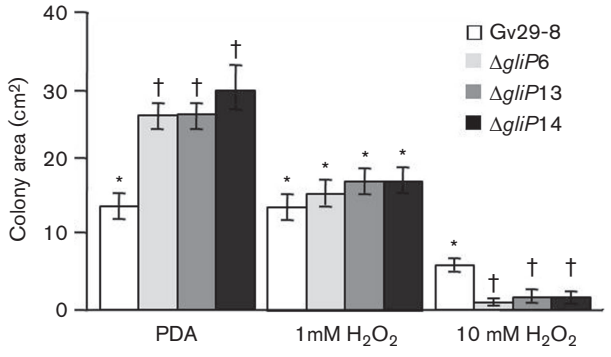

Fig. 4. Effect of oxidative stress on Δ*gliP* mutants. Gv29-8, Δ*gliP*6, Δ*gliP*13 and Δ*gliP*14 were inoculated on PDA plates supplemented with 1 or 10 mM H_2O_2 or on PDA as a control. The colony size was determined 48 h after inoculation. The bars depict the colony area ± SD from three independent experiments. In each treatment, different symbols (*, †) indicate significant differences with a $P<0.01$ according to Fisher's PLSD test.

The role of gliotoxin in biocontrol of soil-borne plant diseases was earlier examined by obtaining non-producing mutants through classical chemical mutagenesis (Wilhite & Straney, 1996; Howell & Stipanovic, 1995). 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

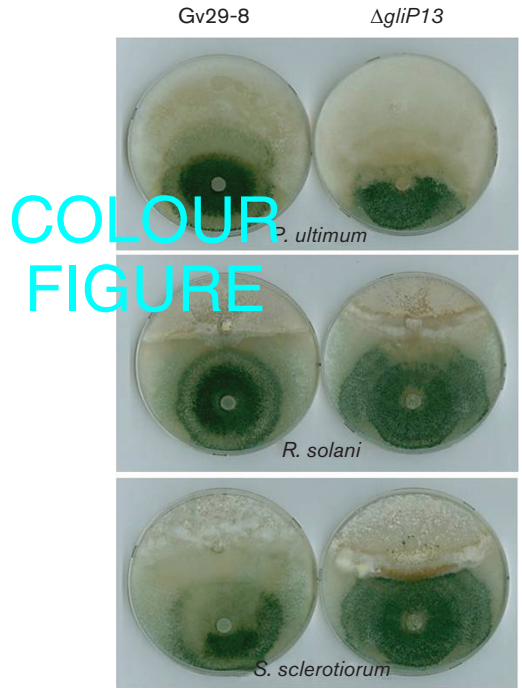

Fig. 5. Gliotoxin biosynthesis differentially contributes to the antagonistic effect of Gv29-8 against *P. ultimum*, *S. sclerotiorum* and *R. solani*. Dual cultures of strains Gv29-8 and Δ*gliP*13, and the pathogens *P. ultimum*, *S. sclerotiorum* and *R. solani*, on PDA medium. Plates were incubated at 25 °C for 7 days.

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 linage 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 effects 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*

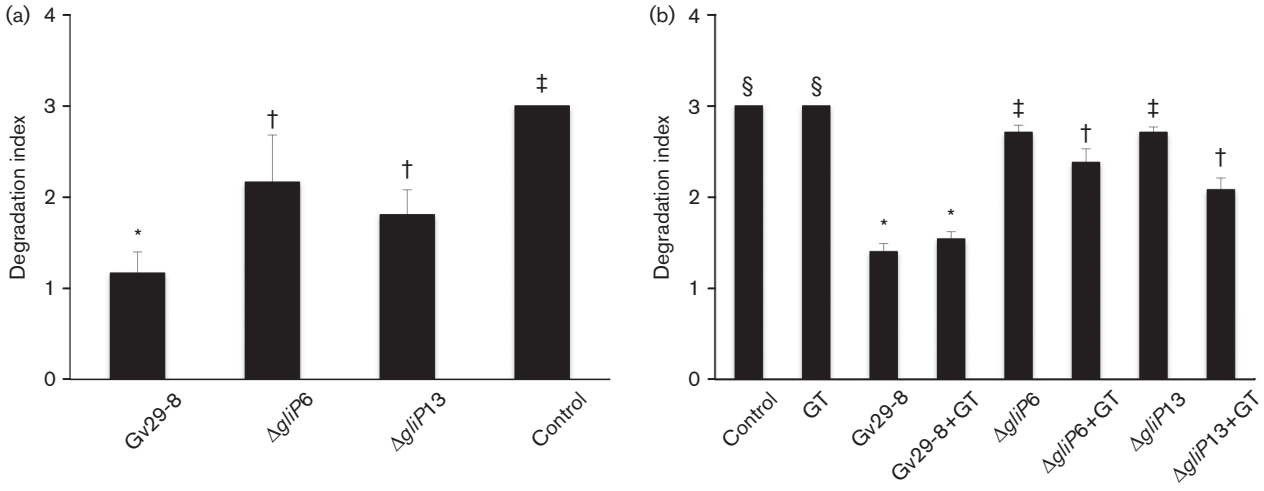

Fig. 6. Sclerotia susceptibility assay. (a) Degradation index after 11 days incubation in the presence of the different strains of *T. virens*; (b) sclerotia were dipped in a gliotoxin solution (20 mg ml^{-1} in methanol) or in methanol as a control, prior to exposure to *T. virens*. The bars represent the mean value ± SD of three independent experiments; different symbols (*, †, ‡, §) represent significant differences ($P \leqslant 0.01$) according to Fisher's PLSD test.

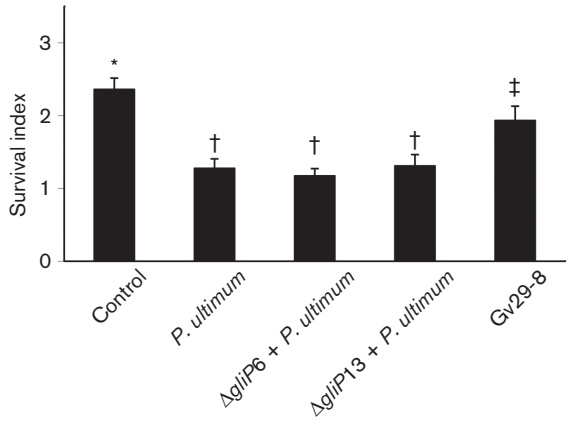

Fig. 7. Biocontrol assay of *T. virens* WT (Gv29-8) and Δ*gliP* mutants against *P. ultimum* in cotton. Each bar represents the survival index determined according to the procedure described in Methods. Treatments are as follows: control, negative control (no pathogen, no *Trichoderma*); *P. ultimum*, positive control (pathogen present, no *Trichoderma*); Δ*gliP*6 + *P. ultimum*, pathogen and strain Δ*gliP*6 present; Δ*gliP*13 + *P. ultimum*, pathogen and strain Δ*gliP*13 present; Δ*gliP*14 + *P. ultimum*, pathogen and strain Δ*gliP*14 present; Gv29-8 + *P. ultimum*, pathogen and *T. virens* Gv29-8 present. Each bar represents the survival index from three replicates with 10 plants each from 3 independent experiments with SE bars. The different symbols (*, †, ‡) represent significant differences ($P<0.01$) according to Fisher's PLSD test.

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

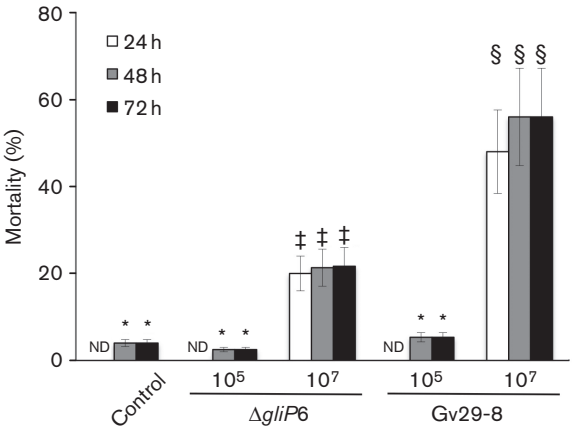

Fig. 8. Mortality of larvae of the wax moth *G. mellonella*. Five microlitres conidial suspension (1×10^5 or 1×10^7 conidia ml^{-1}) of the WT or mutant strains were injected into *G. mellonella* larvae at the final instar stage and mortality was assessed daily. The different symbols (*, ‡, §) represent significant differences ($P<0.01$) according to Fisher's PLSD test. ND, ?????????????.

(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 with *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 broken via reduction is auto-oxidized into its disulphide 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 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 to 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_2O_2, the precise mechanisms are not fully understood. In *A. fumigatus* it was demonstrated that normal expression of *gli*K is important for gliotoxin accumulation and to cope with oxidative stress (Gallagher *et al.*, 2012). In *T. virens*, the sensitivity of the *gliP*-disrupted mutants to oxidative stress may be related to a co-regulation of *gliP* and ROS-detoxifying mechanisms or to a direct effect of gliotoxin itself on H_2O_2. Further investigations will be necessary to provide a better understanding of the mechanisms involved in gliotoxin-mediated H_2O_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 to be used in biocontrol applications

as a formulated product or in transgenic or overexpressing strains needs to be assessed in field trials. The anticancer effects of this reactive compound warrants further examination as ETPs and ETP-like alkaloids have exhibited potent anticancer 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 (Flygare *et al.*, 2013; Teicher & Doroshow, 2012). Further studies of *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 field crops as well as mammalian diseases.

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