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Gliotoxin effects on fungal growth: Mechanisms and exploitation

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ABSTRACT

Although initially investigated for its antifungal properties, little is actually known about the effect of gliotoxin on *Aspergillus fumigatus* and other fungi. We have observed that exposure of *A. fumigatus* to exogenous gliotoxin (14 μg/ml), under gliotoxin-limited growth conditions, results in significant alteration of the expression of 27 proteins (up- and down-regulated >1.9-fold; $p < 0.05$) including *de novo* expression of Cu, Zn superoxide dismutase, up-regulated allergen Asp f3 expression and down-regulated catalase and a peroxiredoxin levels. Significantly elevated glutathione GSH levels ($p < 0.05$), along with concomitant resistance to diamide, were evident in *A. fumigatus* Δ*gliT*, lacking gliotoxin oxidoreductase, a gliotoxin self-protection gene. *Saccharomyces cerevisiae* deletents (Δ*sod1* and Δ*yap1*) were hypersensitive to exogenous gliotoxin, while Δ*gsh1* was resistant. Significant gliotoxin-mediated (5 μg/ml) growth inhibition ($p < 0.001$) of *Aspergillus nidulans, Aspergillus terreus, Aspergillus niger, Cochliobolus heterostrophus* and *Neurospora crassa* was also observed. Growth of *Aspergillus flavus, Fusarium graminearum* and *Aspergillus oryzae* was significantly inhibited ($p < 0.001$) at gliotoxin (10 μg/ml), indicating differential gliotoxin sensitivity amongst fungi. Re-introduction of *gliT* into *A. fumigatus* Δ*gliT*, at a different locus (*ctsD*; AFUA_4G07040, an aspartic protease), with selection on gliotoxin, facilitated deletion of *ctsD* without use of additional antibiotic selection markers. Absence of *ctsD* expression was accompanied by restoration of *gliT* expression, and resistance to gliotoxin. Thus, we propose *gliT*/gliotoxin as a useful selection marker system for fungal transformation. Finally, we suggest incorporation of gliotoxin sensitivity assays into all future fungal functional genomic studies.

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1. Introduction

Production of gliotoxin, an epipolythiodioxopiperazine, by *Aspergillus fumigatus* is enabled by a 28 kb gene cluster (*gli*) located on chromosome 6 (Gardiner et al., 2004; Cramer et al., 2006). The molecule has been intensively studied because of its cytotoxic, immuno-inhibitory and apoptotic effects on animal cells (Sutton et al., 1994). Gliotoxin has recently been shown to inhibit angiogenesis and it has been proposed that this effect prevents tissue neovascularisation, impedes the proinflammatory response and contributes to tissue damage (Ben-ami et al., 2009). As a result of disulphide bridge presence, gliotoxin is a redox active molecule and can cycle between the oxidised and reduced forms depending on the cellular redox state (Waring et al., 1995). In an elegant set of experiments, Bernardo et al. (2003) demonstrated that the oxidised form of gliotoxin is taken up by mammalian cells, undergoes glutathione (GSH)-mediated reduction to the reduced form, which ultimately re-oxidises upon depletion of intracellular GSH and is effluxed from the cell. Deletion of *gliZ* (Bok et al., 2006) and *gliP* (Cramer et al., 2006; Kupfahl et al., 2006), *gliT* (Schrettl et al., 2010; Scharf et al., 2010) and *gliG* (Davis et al., 2011) respectively, abolishes gliotoxin production by *A. fumigatus*. Indeed, Cramer et al. (2006) demonstrated that exogenous gliotoxin controlled the expression of the *gli* cluster in *A. fumigatus* and thereby regulated its own production.

Chamilos et al. (2008) studied the effect of gliotoxin on *Saccharomyces cerevisiae*, using a library of single-gene mutants (4787 strains), in an attempt to further elucidate mechanisms of gliotoxin cytotoxicity and identify novel drug targets in eukaryotic cells. Overall, 10 mutants exhibited increased resistance to gliotoxin while 3 were statistically more sensitive to exogenous gliotoxin, compared to wild-type. Increased resistance to gliotoxin was observed in strains lacking genes encoding metabolic, vesicular transport, DNA damage repair and unknown function functions, while increased sensitivity was evident when Cys3, a cystathionine gamma-lyase and Mef1 a transcriptional elongation factor of mitochondrial origin were absent.

Rohlfs et al. (2007) demonstrated that toxic secondary metabolites produced by *Aspergillus nidulans*, under control of the global regulator *laeA*, significantly discouraged feeding by the fungivore,

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Folsomia candida and proposed that fungivory may select for secondary metabolite biosynthesis in fungi. This led Kwon-Chung and Sugui (2009) to note that production of secondary metabolites might improve the survival chances of fungi. Moreover, although secondary metabolite production may also be influenced by competition between different fungal species, until recently, it was not known if secondary metabolites produced by *Aspergilli* conferred a growth competitive advantage in the presence of related species (Losada et al., 2009). Although these authors identified a number of secreted metabolites, which may contribute to improved survival, no information with respect to individual metabolite potency, or the effect on non-*Aspergillus spp*. was presented. Losada et al. postulated that resistance genes would be required to allow a toxin producer to grow in the presence of its metabolite weaponry and it has now been shown that *gliT* in the *gli* cluster mediates self-protection against exogenous gliotoxin in *A. fumigatus* (Schrettl et al., 2010; Scharf et al., 2010) whereby a *gliT*-deficient strain was unable to grow in the presence of gliotoxin. In fact, *gliT* is the key resistance gene against gliotoxin in *A. fumigatus* and Schrettl et al. demonstrated that Δ*gliT* complementation was possible using selection on gliotoxin-containing media without additional antibiotics (e.g., hygromycin or phleomycin). However, use of *gliT* re-introduction into *A. fumigatus* Δ*gliT*, and subsequent selection in gliotoxin presence, for the targeted deletion of unrelated genes has not been demonstrated.

Thus, a new paradigm has emerged where the effects, and role, of gliotoxin in *A. fumigatus*, in particular, and fungi generally, requires investigation: as it may represent a novel probe to further our understanding of the function of ETPs, and redox control mechanisms, in fungi. In addition, exploitation of the effects of gliotoxin to develop a new fungal selection marker system may be feasible.

2. Materials and methods

2.1. Extraction of gliotoxin from culture filtrates

A. fumigatus ATCC26933 (1×10^5 cfu/ml) was grown for 24 h in either Minimal Essential Medium (plus 5% (v/v) fetal calf serum (MEM/FCS)) or Sabouraud media (25 ml cultures), at 37 °C with shaking at 200 rpm. Supernatants were separated by filtration and an equal volume of extraction buffer (ethyl acetate:chloroform:methanol, 3:2:1) was added. After overnight incubation at 4 °C, filtrates were centrifuged and organic layers removed and evaporated to dryness. Dried extracts were reconstituted in 200 μl HPLC grade Methanol and stored at −70 °C until assay.

2.2. RP-HPLC analysis

Gliotoxin was detected using a reversed phase HPLC (Spectra-Physics). Gradient elution was performed with Solvent A consisting of 0.1% (v/v) trifluoroacetic acid in 5:95 acetonitrile:HPLC grade water (Sigma–Aldrich) and Solvent B consisting of 0.1% (v/v) trifluoroacetic acid in 99.9% acetonitrile. Gliotoxin extracts (20 μl) were injected onto the C_{18} column (Hewlett Packard). All cultures were grown up in triplicate and each one analysed in duplicate. Average values were compared to a standard curve (0–1.0 μg gliotoxin) and gliotoxin quantified as micrograms of gliotoxin per milligram of mycelia produced ±standard deviation.

2.3. Protein extraction

A. fumigatus ATCC26933 (1×10^5 cfu/ml) was grown for 24 h in Sabouraud media (50 ml cultures), at 37 °C with shaking at 200 rpm. After 24 h, gliotoxin (700 μg) dissolved in methanol was added to the cultures ($n = 3$). As a control, methanol only was added to parallel cultures ($n = 3$). After 4 h incubation, mycelia were harvested, filtered under pressure, washed with PBS and resuspended in lysis buffer (100 mM Tris–HCl, 50 mM NaCl, 20 mM EDTA, 10% (v/v) glycerol, 30 mM DTT, 1 mM PMSF and 1 μg/ml pepstatin A pH 7.5; 3 ml of lysis buffer per gram of mycelia). Lysis was accomplished by grinding in liquid N_2 followed by brief sonication on ice. Mycelial lysates were centrifuged (10,000*g*; 30 min) to remove cell debris and the subsequent supernatants analysed by 2D-PAGE following TCA/acetone precipitation (Carberry et al., 2006).

2.4. Protein quantification

Protein was quantified using Bradford reagent (BioRad Laboratories) with BSA used as a standard protein.

2.5. Two-dimensional electrophoresis

Extracts containing 250 μg protein were resuspended in 8 M Urea, 2 M Thiourea, 4% (w/v) CHAPS, 1% (v/v) Triton-X-100, 10 mM Tris-HC1, 65 mM DTT, 0.8% pH 4–7 carrier ampholytes and loaded onto Immobiline Dry strips (GE Healthcare) in the pH range 4–7. Following IEF on an IPGphor II, gels were equilibrated in reducing buffer (50 mM Tris–HCl, 6 M Urea, 2% (w/v) SDS, 30% (v/v) Glycerol, 2% (w/v) DTT, pH 6.8) for 20 min followed by equilibration in alkylation buffer (50 mM Tris–HCl, 6 M urea, 2% (w/v) SDS, 30% (v/v) glycerol, 2.5% (w/v) iodoacetamide, pH 6.8) for a further 20 min. The equilibrated strips were placed on homogenous 12% SDS–PAGE gels and electrophoresed overnight at 100 V using a Protean Xi-II Cell (Bio-Rad Laboratories). Resulting gels were stained with Coomassie Brilliant Blue R and scanned using a Typhoon Trio Variable Mode Imager (GE Healthcare, Freiburg, Germany). Three replicate gels of each, treated and untreated were submitted for analysis using the Image Master Platinum software (GE Healthcare, Freiburg, Germany) to identify differentially regulated spots. The protein spots of interest on each gel were detected, normalised, edited and manually matched to a reference gel (Fig. 1A).

2.6. MALDI-ToF mass spectrometry

Selected proteins were excised from 2D-PAGE gels using an automated spot cutter (GE Healthcare, Freiburg, Germany), digested with trypsin and analysed as previously described (Carberry et al., 2006). Mass spectrometry was carried out using an Ettan™ MALDI-ToF mass spectrometer (GE Healthcare, Freiburg, Germany), internal calibrants, Angiotensin III (Sigma–Aldrich) and ACTH fragment 18–39 (Sigma–Aldrich), were used to calibrate all spectra. Protein identification was carried out either by m/z data interrogation of a (I) local FASTA version of the annotated *A. fumigatus* genome available at http://www.cadre-genomes.org.uk/ and (II) Mascot™ nrNCBI database (Mabey et al., 2004).

2.7. GSH/GSSG and superoxide dismutase activity determination

Intracellular glutathione was measured using the method of Thön et al. (2010) and Rahman et al. (2006), with modifications. *A. fumigatus* ATCC26933 and Δ*gliT* (Schrettl et al., 2010), respectively, were cultured for 21 h in AMM before addition of gliotoxin (5 μg/ml final) for 3 h. Mycelia were harvested through miracloth and dried. Mycelia (500 mg) in 5% (w/v) SSA (500 μl) were bead-beaten at 30 Hz for 5 min followed by centrifugation at 12,000*g* for 10 min at 4 °C. Supernatants were removed, covered in tinfoil and neutralised using triethanolamine. All samples were diluted (1/10–1/30) in 125 mM sodium phosphate, 6.3 mM EDTA, pH 7.5 prior to centrifugation at 12,000 g for 10 min at 4 °C. Whole cell

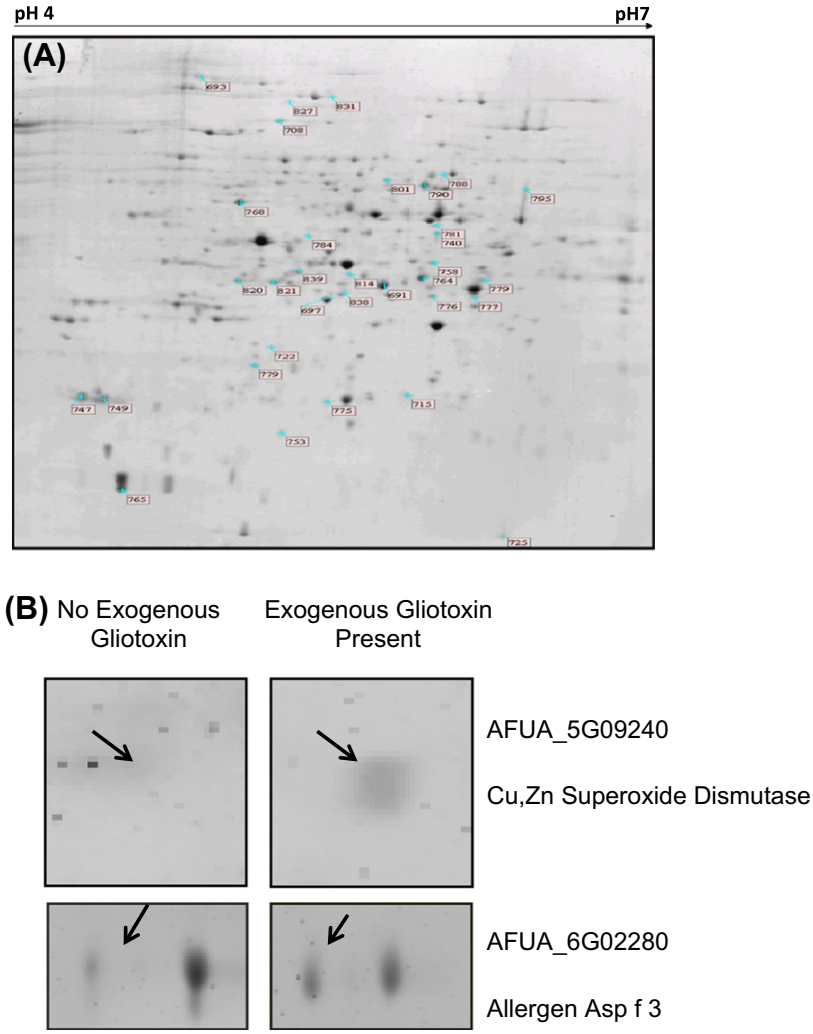

Fig. 1. 2D-PAGE analysis of *A. fumigatus* challenged with exogenous gliotoxin (14 μg/ml) resulted in the identification of 27 proteins that were differentially regulated in response to the exogenous gliotoxin. (A) Proteins were separated across the entire p*I* range of pH 4–7 strips and are present from less than 16 kDa up to over 100 kDa. Numbered spots correspond to protein numbers in Tables 2 and 3. (B) Zoomed image of Cu, Zn superoxide dismutase *de novo* expression and up-regulated Asp f3 expression.

protein was also extracted in the same manner using lysis buffer (100 mM Tris-HCl, 50 mM NaCl, 20 mM EDTA, 10% (v/v) glycerol, 30 mM DTT, 1 mM PMSF, and 1 μg/ml pepstatin A, pH 7.5) and quantified by Bradford Assay. 2-vinylpyridine (2 μl, 1/10 in assay buffer) was added for GSSG determination, incubated at room temperature for 1 h before triethanolamine (6 μl) was added to neutralise the 2-vinylpyridine. All samples were incubated for 10 min at room temperature prior to assay. Then, blanks/standards/samples (20 μl) were added to 96-well microtiter plates followed by 10 mM dithionitrobenzoic acid (DTNB) containing 1 unit glutathione reductase and left for 30 s. NADPH (5 mM) was added to each well, microplates gently shaken and A412 nm measured after 90 s. Superoxide dismutase activity was determined in mycelial protein lysates ($n = 3$ each), obtained following mycelial incubation with and without gliotoxin (14 μg/ml) for 4 h. Superoxide dismutase assays were carried out as described by Raimondi et al. (2010), except that nitroblue tetrazolium was used instead of cytochome *c*.

2.8. Plate assays

For plate assays, 10^3–10^5 freshly harvested conidia of various Aspergillus species, including *A. nidulans*, *A. flavus*, *A. niger*, *A. oryzae*, *A. terreus*, *Neurospora crassa*, *Cochliobolus heterostrophus* and *Fusarium graminearum* were point inoculated on plates containing gliotoxin in a range of 0, 0.1–30 μg/ml. As controls, A. fumigatus wild-type ATCC46645 and Δ*gliT* (Schrettl et al., 2010) were used. The diameter of the fungal colonies was determined after 72 h and normalised to the diameter on plates without the addition of gliotoxin. For experiments utilising *S. cerevisiae*, strain background BY4741 (*MATa: his3Δ1: leu2Δ0: met15Δ0: ura3Δ0*) and deletion derivatives were used throughout. Yeast strains deleted for *SOD1* and *GSH1* were purchased from Euroscarf. Spot growth assays were carried out by diluting an overnight culture of cells in fresh YPD medium to 1×10^6 cells/ml. Cells were then grown to exponential phase to a density of 3×10^6 cells/ml. Cells were then re-suspended in YPD to a density of 5×10^6 cells/ml and transferred to a microtiter plate. Following a series of 1 in 5 dilutions, cells were transferred to YPD plates using a multi-pronged replicator and incubated at 30 °C for 2–3 days. *GSH1* coding region ±500 bp was cloned into pRS315 (*LEU2*) by gap repair and efficacy of the construct confirmed by sequencing.

2.9. ctsD deletion in A. fumigatus ΔgliT

An aspartic protease is encoded by *A. fumigatus ctsD* (AFUA_4G07040; Vickers et al., 2007). To generate *A. fumigatus*

ΔctsD, the bipartite marker technique (Nielsen et al., 2006) was used, with modifications. Briefly, *A. fumigatus* Δ*gliT*^{46645} (Schrettl et al., 2010) was co-transformed with two DNA constructs, each containing an incomplete fragment of the gliotoxin oxidoreductase gene, *gliT* (Schrettl et al., 2010; Scharf et al., 2010) fused to 1.5 kb, and 1.7 kb of *ctsD* 5′ and 3′ flanking sequences, respectively. These marker fragments shared a 242 bp overlap within the *gliT* cassette, which served as a potential recombination site during transformation. Two rounds of PCR generated each fragment (Table 1). First, each flanking region was amplified from *A. fumigatus* ATCC46645 genomic DNA using primer o*gliT*-1 and o*gliT*-2 for flanking region A (1.5 kb), and o*gliT*-3 and o*gliT*-4 for flanking region B (1.7 kb). After purification, the fragments were digested with *Rsr*II and *Psi*I, respectively. *gliT* was PCR amplified from *A. fumigatus* ATCC46645 genomic DNA and inserted into TOPO cloning vector (Invitrogen). *gliT* selection marker was removed from the TOPO cloning vector following double restriction digest with *Rsr*II and *Psi*I and ligated with the two flanking regions A and B described above. Subsequently, two overlapping fragments were amplified from the ligation products using primers o*gliT*-5 and o*gliT*-6 for fragment C (2.1 kb) and primers o*gliT*-7 and o*gliT*-8 for fragment D (2.2 kb). For generation of Δ*ctsD*, *A. fumigatus* Δ*gliT*^{46645} protoplasts were transformed simultaneously with the overlapping fragments C and D, as described by Schrettl et al. (2010). Subsequent selection was performed on *Aspergillus* Minimal Medium (AMM) with 0.7% (w/v) agar including 1 M sucrose as an osmotic stabiliser and containing gliotoxin (30 μg/ml). Southern analyses, to confirm *gliT* insertion at the *ctsD* locus was performed using a digoxigenin (DIG)-labelled probe, generated using primers o*gliT*-5 and o*gliT*-2, against the 5′-flanking region of c*tsD* following *Afl*III restriction digest of *A. fumigatus* transformant genomic DNA. Further Southern analyses, via *Xba*I digestion of *A. fumigatus* transformant genomic DNA, to confirm the loss of *ctsD* and acquisition of the *gliT*, were undertaken using DIG-labelled c*tsD* and *gliT* probes prepared by PCR using primers o*gliT*-9 and o*gliT*-10 for *ctsD* and o*gliT*-11 and o*gliT*-12 for the *gliT* probe, respectively. RT-PCR analyses were performed to confirm loss of *ctsD* expression and assessment of g*liT* expression using primers o*gliT*-13 and o*gliT*-14 for *ctsD* mRNA analysis and o*gliT*-7 and o*gliT*-6 for *gliT* mRNA analysis.

Table 1
Primers used in this study.

Primer	Sequence 5′–3′	Use(s)
ogliT-1	AAAGGCCATGCGAGCTACTA	Flanking region A
ogliT-2[a]	CCCGGTCCGAATCTATGGTCGC	Flanking region A, 5′-flanking ctsD probe
ogliT-3[b]	CCTTATAAGCATTGATGGGTC	Flanking region B
ogliT-4	CTCTCTATCAGCGGGAAAAC	Flanking region B
ogliT-5	CGCCAATGTGACAGAGATCA	Overlapping fragment C, 5′-flanking ctsD probe
ogliT-6	GACACCCACGGTCTCCTG	Overlapping fragment C, gliT RT-PCR
ogliT-7	ACTCCACCATCCAGTTCCAG	Overlapping fragment D, gliT RT-PCR
ogliT-8	AGTCCGGATTCGACGTACTG	Overlapping fragment D
ogliT-9	TTTCTCTCGCCTCCACAGTT	ctsD Probe
ogliT-10	TTGTAGCTAAGGCCCGAGAA	ctsD Probe
ogliT-11	CTACTCTCGCGCATACCACA	gliT Probe
ogliT-12	GCTCCTGATCGAGACGAAAC	gliT Probe
ogliT-13	GAATGGTACGCCTTGTCGTT	ctsD RT-PCR
ogliT-14	TTGTAGCTAAGGCCCGAGAA	ctsD RT-PCR
Calm F[c]	CCGAGTACAAGGAAGCTTTCTC	calm RT-PCR
Calm R[c]	GAATCATCTCGTCGATTCGTCGT CTCAGT	calm RT-PCR

[a] *Rsr*II restriction site underlined.
[b] *Psi*I restriction site underlined.
[c] Burns et al. (2005).

3. Results

3.1. Determination of media-dependence of gliotoxin production in A. fumigatus

The concentration of gliotoxin in Sabouraud and MEM/FCS culture supernatants was determined to be 1.35 ± 0.3 μg and 23.65 ± 1.3 μg per mg mycelia, respectively. Thus, a 17-fold difference in gliotoxin production was evident which was directly related to culture medium composition. Moreover, this observation facilitated evaluation of the effect of increased extracellular gliotoxin levels on *A. fumigatus*, against a background of minimal gliotoxin biosynthesis and secretion.

3.2. Exogenous gliotoxin alters specific protein expression in A. fumigatus

Following exposure of *A. fumigatus* in Sabouraud media (24 h) to control conditions (methanol) and elevated gliotoxin (700 μg in methanol) addition, mycelia were harvested after 4 h and lysed, followed by 2D-PAGE, comparative protein expression analysis and MALDI-ToF mass spectrometry to facilitate protein identification (Fig. 1A and B, Tables 2 and 3). Overall, perturbation in the expression of at least 27 distinct proteins was detected, comprising of both up-regulation (18; 64%) and down-regulation (9; 33%) in response to gliotoxin exposure. *De novo* expression of a Cu, Zn superoxide dismutase (AFUA_5G09240; SOD; Fig. 1B), a short chain dehydrogenase (AFUA_4G08710) and a eukaryotic translation elongation factor β1 subunit (AFUA_1G11190) were also clearly observed following gliotoxin exposure (Table 2). Interestingly, superoxide dismutase activity was significantly elevated ($p < 0.03$) from 0 (pre-exposure) to 92 ± 41 U/mg in cultures exposed to gliotoxin, in accordance with proteomic data. Moreover, reactive oxygen species (ROS) production, also accompanied mycelial exposure to gliotoxin (data not shown). Three proteins (putative adenine phosphoribosyltransferase, uridylate kinase and an aminopeptidase) exhibited between 10–20-fold increased expression, while three proteins: Vip1, a putative thiazole biosynthesis enzyme and allergen Asp f3 (Fig. 1B) were up-regulated 9.49, 7.03 and 5.8-fold, respectively. In addition, expression of eight other proteins, including a putative heat shock protein, Sti1, and thiamine biosynthesis protein, Nmt1, was induced by 1.9–3.0-fold. Overall, identification of proteins whose expression was up-regulated by gliotoxin exposure was confirmed by overall sequence coverage ranging from 17.6–49.3%. The expression of nine proteins was found to be significantly ($p < 0.05$) down-regulated following gliotoxin exposure (Table 3). Mycelial catalase (AFUA_3G02270) expression reduced by over 40-fold, while that of ATP-citrate lyase (AFUA_6G10660) and fumaryl acetoacetate hydrolase (FahA; AFUA_2G04230) diminished by over 26 and 21-fold, respectively. The expression of the remaining six proteins, including antioxidant protein LsfA (peroxredoxin; AFUA_4G08580) and dehydrogenases/oxidoreductases, ranged from a 4.2–9.6-fold reduction, compared to control conditions.

3.3. Intracellular GSH levels are significantly altered in A. fumigatus ΔgliT

GSH levels were significantly elevated ($p < 0.05$) in *A. fumigatus* Δ*gliT* compared to wild-type (Fig. 2A) which suggests that elevated GSH may potentiate the autotoxic effects of gliotoxin. Differences in intracellular GSH levels remained significantly different ($p < 0.05$), in *A. fumigatus* wild-type and Δ*gliT*, even in the presence of gliotoxin (5 μg/ml). However addition of gliotoxin, to either *A. fumigatus* wild-type or Δ*gliT*, resulted in a diminution in intracellular GSH

Table 2
Selected proteins undergo significantly increased expression[a] following exposure of *A. fumigatus* ATCC26933 to gliotoxin.[b] Proteins are listed according to fold increase in expression.

Proposed/actual function	Fold increase	% Sequence coverage	Mass (Da)	CADRE I.D.[c]	Spot No.
Cu, Zn superoxide dismutase	(+) Unique	33.5	16,361	AFUA_5G09240	725
Short chain dehydrogenase	(+) Unique	29.6	30,928	AFUA_4G08710	779
Eukaryotic translation elongation					
Factor 1β subunit	(+) Unique	25.9	30,142	AFUA_1G11190	714
Adenine phosphoribosyltransferase	20.5	33.6	23,621	AFUA_7G02310	715
Uridylate kinase	10.3	33.2	25,560	AFUA_7G03990	722
Aminopeptidase	10.1	26	106,227	AFUA_4G09030	693
Vip1 protein[d,e]	9.5	24.2	28,265	AFUA_2G10030	777
Vip1 protein[d,e]	7.0	44.2	28,265	AFUA_2G10030	776
Thiazole biosynthesis enzyme	7.0	27.7	35,492	AFUA_6G08360	814
Allergen Asp F3	5.8	30.4	18,453	AFUA_6G02280	765
Nitroreductase family protein	3.0	49.3	24,314	AFUA_5G09910	753
Unknown function protein[d,e]	2.9	30.8	25,439	AFUA_5G14680	749
Heat shock protein (Sti1), putative	2.9	17.6	65,031	AFUA_7G01860	708
Allantoicase	2.7	21.3	46,273	AFUA_3G12560	740
Mitochondrial processing					
Peptidase β subunit	2.7	31.5	53,270	AFUA_1G14200	768
Glycerol dehydrogenase	2.5	25.8	36,828	AFUA_4G11730	691
Unknown function protein[d,e]	2.1	37.9	25,439	AFUA_5G14680	747
Thiamine biosynthesis protein Nmt1	1.9	33.9	38,323	AFUA_5G02470	764
Phosphoglycerate kinase	1.9	34.2	44,761	AFUA_1G10350	758

[a] $p < 0.05$.
[b] GliT (AFUA_6G09740) expression was also significantly increased, 2.5–3.5-fold (Schrettl et al., 2010).
[c] CADRE I.D: CADRE Identification (Mabey et al., 2004).
[d,e] Vip1 protein and unknown function protein were each detected in two adjacent protein spots, respectively.

Table 3
Selected proteins undergo significantly decreased expression[a] following exposure of *A. fumigatus* ATCC26933 to gliotoxin. Proteins are listed according to fold decrease in expression.

Proposed/actual function	Fold decrease	% Sequence coverage	Mass (Da)	CADRE I.D.[b]	Spot No.
Mycelial catalase 1	−41.6	20.3	79,910	AFUA_3G02270	827
ATP-citrate lyase	−26.2	30.7	52,918	AFUA_6G10660	790
Fumarylacetoacetate hydrolase FahA	−21.2	18.3	46,817	AFUA_2G04230	781
Aminopeptidase B	−9.6	7.9	108,707	AFUA_5G04330	831
Aldo–keto reductase family 7 protein	−9.0	37.2	35,489	AFUA_5G02020	697
Aldehyde dehydrogenase	−8.9	25.6	53,759	AFUA_7G01000	795
Alcohol dehydrogenase, Zn containing	−8.5	47.1	37,854	AFUA_1G04620	779
Anti-oxidant protein LsfA peroxiredoxin	−6.9	31.9	23,393	AFUA_4G08580	775
GMC oxidoreductase	−4.2	17.6	72,151	AFUA_3G01580	706

[a] $p < 0.05$.
[b] CADRE I.D, CADRE identification (Mabey et al., 2004).

levels (nmol/mg protein), in both strains, without a concomitant increase in GSSG. Although not statistically significant, these trends were reproducible, and suggest formation of mixed gliotoxin-GSH disulphides. Moreover, the GSH/GSSG ratio in *A. fumigatus* ATCC26933 was 8.15, while it was increased to 47.5 in *A. fumigatus* Δ*gliT*. This dramatic increase in ratio in *A. fumigatus* Δ*gliT* must be caused by the significant increase ($p < 0.05$) in GSH levels as there was no observed difference in the GSSG levels. Relatedly, *A. fumigatus* Δ*gliT* was significantly more resistant to the effects of diamide, a GSH scavenger, than either wild-type or *gliT*^{C} strains (Fig. 2C), thereby providing complementary support for the observed GSH levels. Future work will investigate the effects of gliotoxin on GSH reductase and peroxidase activities, respectively, to further elucidate the interplay between GliT, gliotoxin and these catalytic functions.

3.4. Gliotoxin disrupts the intracellular redox balance in S. cerevisiae

Fig. 3A shows that growth of *S. cerevisiae* BY4741 (wild-type) is impeded by exposure to exogenous gliotoxin (8 μg/ml) and that *S. cerevisiae* Δ*sod1* and Δ*yap1* exhibit increased hypersensitivity to exogenous gliotoxin compared to the wild-type strain. This observation indicates that gliotoxin induced an oxidative stress in *S. cerevisiae* and may cause superoxide formation. Importantly, compared to wild-type, no growth difference was observed when *S. cerevisiae* Δ*ctt1*, Δ*gsh2* or Δ*glr1* were exposed to identical gliotoxin concentrations (data not shown). To further explore the mechanism underlying gliotoxin-mediated growth inhibition, *S. cerevisiae* Δ*gsh1*, which is deficient in intracellular GSH (Lee et al., 2001), and Δ*gsh1*:*GSH1*, the complemented strain, were exposed to gliotoxin. It can be seen in Fig. 3B that *S. cerevisiae* Δ*gsh1* exhibited increased resistance to exogenous gliotoxin (16 μg/ml) compared to wild-type and that re-introduction of *GSH1* in the deletion strain restored gliotoxin sensitivity. This observation suggests that decreased intracellular GSH levels may protect *S. cerevisiae* against gliotoxin by attenuating production of intracellular reduced gliotoxin (gliotoxin-$(SH)_2$).

3.5. Gliotoxin inhibits fungal growth

The proposed use of *gliT* as a selection marker during transformation in fungi relies on the sensitivity of these fungal species to gliotoxin (Schrettl et al., 2010). Hence, it was necessary to test the susceptibility of potential target fungi towards treatment with

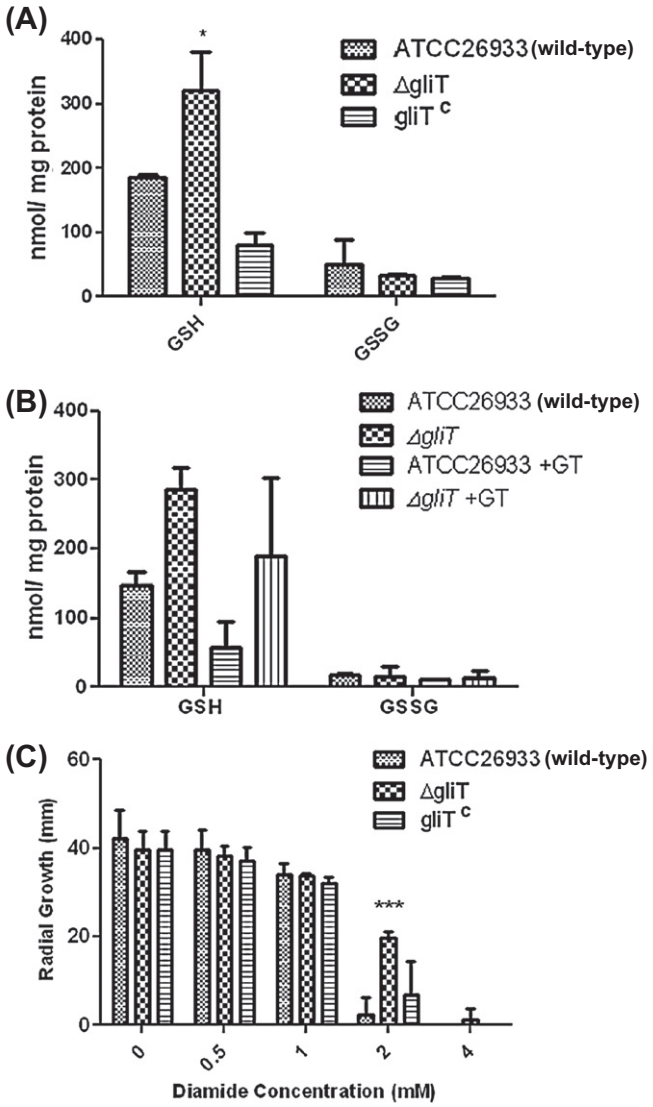

Fig. 2. Assessment of redox status in *A. fumigatus ΔgliT*. (A) Significantly elevated GSH is evident in *A. fumigatus ΔgliT* compared to wild-type and *A. fumigatus gliT*^{c}. This may sensitise the mutant to gliotoxin. (B) GSH and GSSG levels in *A. fumigatus* ATCC26933 and Δ*gliT* in the absence and presence of gliotoxin (GT) for 3 h. Under basal conditions, there is a significant increase ($p < 0.05$) in GSH levels in *A. fumigatus* Δ*gliT* compared to *A. fumigatus* ATCC26933. Gliotoxin addition depletes GSH in both strains without affecting the significant difference ($p < 0.05$) between GSH levels in both strains. (C) *A. fumigatus ΔgliT* is significantly more resistant to the GSH-scavenging agent, diamide. This is most likely a consequence of elevated intracellular GSH. In both cases, complementation restored the wild-type phenotype.

gliotoxin. This was achieved by performing plate assays containing different concentrations of gliotoxin. For plate assays, 10^3–10^5 freshly harvested conidia of various *Aspergillus* species, including *A. nidulans, A. flavus, A. niger, A. oryzae, A. terreus, N. crassa, C. heterostrophus* and *F. graminearum* were point inoculated on plates containing gliotoxin in a range of 0, 0.1–30 μg/ml. Statistically significant ($p < 0.001$ using Bonferronis Multiple Comparison Test), gliotoxin-mediated growth inhibtion of *A. nidulans, A. terreus, A. niger, C. heterostrophus* and *N. crassa* was observed at 5 μg/ml gliotoxin (Fig. 4A and B). This establishes all of these strains as suitable for transformation with *gliT* to facilitate transformant selection on gliotoxin. Growth of *A. flavus, F. graminarium* and *A. oryzae* was significantly inhibited ($p < 0.001$) at 10 μg/ml gliotoxin (Fig. 4). Again, this indicates suitability of these as target strains for transformation with *gliT* to facilitate transformant selection on gliotoxin, albeit necessitating the use of higher concentrations of gliotoxin to facilitate selection.

3.6. Targeted gene deletion in A. fumigatus can be detected by recovery of gliotoxin resistance

A. fumigatus ATCC46645 *ctsD* (AFUA_4G07040) encodes an aspartic protease which was found to be expressed in minimal and proteinaceous media only, and does not play any role in gliotoxin metabolism (Vickers et al., 2007; Gardiner and Howlett, 2005). After confirmation of *ctsD* expression in gliotoxin-sensitive *A. fumigatus* Δ*gliT*^{46645}, targeted deletion of *ctsD* was undertaken using a bipartite strategy (Nielsen et al., 2006) whereby *gliT* introduction at the *ctsD* locus (Fig. 5A), followed by selection of transformants on gliotoxin-containing media (30 μg/ml) was attempted (Fig. 5B). Southern blot analysis of *A. fumigatus* ATCC 46645 or Δ*gliT*^{46645}, using a probe for the 5′ flanking region of *ctsD*, yielded a fragment size of 3530 bp at the *ctsD* locus, whereas transformants ($n = 10$) which had recovered gliotoxin resistance yielded a fragment at 2508 bp thereby confirming disruption of *ctsD*. Furthermore, a fragment of 4832 bp, which contained the *ctsD* open reading frame was absent in all 10 transformants when a *ctsD* coding region probe was employed for Southern analysis (Fig. 5C). Concomitant re-introduction of the *gliT* coding sequence at the *ctsD* locus, detected using a *gliT*-specific probe, in *A. fumigatus* Δ*gliT*, was evident as shown by appearance of a fragment at 5102 bp (corresponding signals for *A. fumigatus* ATCC 46645 and Δ*gliT*^{46645}, were 6403 bp and no signal, respectively) (Fig. 5D). Both *gliT* and *ctsD* expression was evident in *A. fumigatus* ATCC 46645, however as assessed by RT-PCR, *ctsD* expression was not evident in *A. fumigatus* Δ*ctsD*^{46645} but was detectable in *A. fumigatus* Δ*gliT*^{46645} (Fig. 5E). Conversely, *gliT* was expressed in *A. fumigatus* Δ*ctsD*^{46645} which confirmed that successful *ctsD* deletion using *gliT* re-introduction into *A. fumigatus* Δ*gliT*^{46645}, with selection for gliotoxin resistance, had occurred (Fig. 5E).

4. Discussion

Little is known about the effect of gliotoxin on, or in, fungi. Analysis of *A. fumigatus* culture supernatants revealed gliotoxin production to be media specific, with a 17-fold concentration difference between two specific culture media. 2D-PAGE analysis of *A. fumigatus* mycelia challenged with exogenous gliotoxin for 4 h after 24 h growth in Sabouraud media (low endogenous gliotoxin production) led to the identification of 27 differentially-regulated proteins, 18 which exhibited increased expression, and 9 which underwent decreased expression in the presence of gliotoxin. Only proteins with a *t*-test score of $p \leqslant 0.05$ were accepted as significant. Our data further demonstrates dysregulation of intracellular GSH levels in the absence of *A. fumigatus gliT*, a possible consequence of which is elevated sensitivity to exogenous gliotoxin. Because of the increased sensitivity or resistance of *S. cerevisiae* gene deletants to gliotoxin, along with sensitivity of a range of fungal species to gliotoxin, we suggest gliotoxin exposure as a standard phenotypic screen in functional genomic studies. Whether gliotoxin is primarily an antifungal, or a protective molecular species, is discussed below – along with the potential for *gliT*/gliotoxin as an alternative selection marker system for fungal transformation.

Cu, Zn superoxide dismutase (Cu, Zn SOD), (AFUA_5G09240) was expressed *de novo* in the presence of exogenous gliotoxin in *A. fumigatus*. Interestingly, gliotoxin has been shown to produce superoxide radicals in mast cells (Niide et al., 2006), and the *de novo* expression of Cu, Zn SOD suggests that gliotoxin addition may have an identical effect in *A. fumigatus*. Moreover, Cu, Zn SOD was previously identified by Lessing et al. (2007), to be

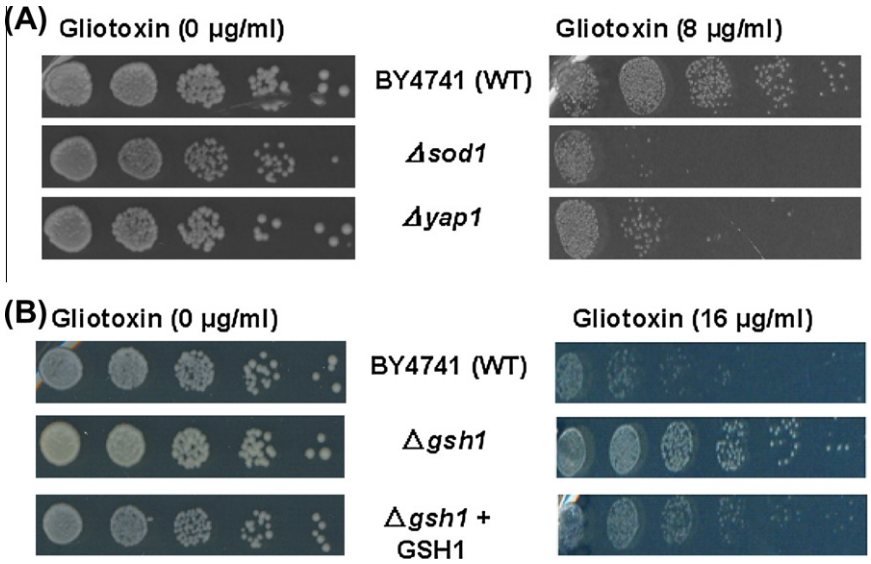

Fig. 3. Effect of gliotoxin on *S. cerevisiae* BY4741 (wild-type), oxidative stress and glutathione-deficient mutants. (A) Absence of *sod1* and *yap1* result in increased sensitivity to exogenous gliotoxin. (B) *S. cerevisiae* sensitivity to gliotoxin is dependent on *GSH1* presence.

involved in defence against oxidative radicals in *A. fumigatus*. Thus, although not essential for virulence (Lambou et al., 2010), the enzyme appears to play a role in ROS and protection against gliotoxin. Interestingly, SOD expression was also increased in *A. flavus* during the production of another mycotoxin, aflatoxin (O'Brian et al., 2007) and production of aflatoxin has been linked with greater oxygen requirements and high levels of ROS (Narasaiah et al., 2006) as has also been suggested for gliotoxin (Watanabe et al., 2004). *A. fumigatus* SOD has been shown to be reactive to sera from aspergillosis patients (Holdom et al., 1996), and is present on the cell wall and in culture media. This may show a possible protective role against the oxidative burst mechanism of neutrophils in response to *A. fumigatus* infection (Hamilton et al., 1996). Bruns et al. (2010) also noted that Cu, Zn SOD gene expression was significantly up-regulated in *A. fumigatus* during biofilm formation. *De novo* expression of eukaryotic elongation factor 1β subunit (AFUA_1G11190) was also observed in *A. fumigatus* mycelia. This protein was previously identified to be abundant in *A. fumigatus* conidia but was found to be relatively weakly immunogenic (Asif et al., 2006). Sugui et al. (2008) demonstrated that short chain dehydrogenase expression (AFUA_4G08710) was significantly up-regulated in conidia, but not hyphae, upon exposure to neutrophils from either normal or CGD donors. Thus, the *de novo* expression of this protein in response to gliotoxin exposure may be due similarities between the intracellular oxidative stress program induced by neutrophils and gliotoxin, respectively. Significantly, cognate mRNA expression of six (adenine phosphoribosyltransferase (APRT), aminopeptidase, Vip1 protein, heat shock protein (Sti1), glycerol dehydrogenase (GldB) and thiamine biosynthesis protein (Nmt1); Table 2) of the 15 proteins whose expression is induced by gliotoxin, was also significantly up-regulated following conidial exposure to airway epithelial cells (Oosthuizen et al., 2011). Elevated expression of two enzymes involved in nucleotide biosynthesis (APRT and uridylate kinase) was also evident upon gliotoxin exposure. Khalaj et al. (2011) noted a 20% reduction in uridylate kinase expression in an annexin disruptant (Δ*anxC4*) and although no functional role was assigned to altered uridylate kinase expression, these authors noted that alterations in stress response related proteins suggested an oxidative stress response in the mutant. Allergen Asp f3 (AFUA_6G02280) is a thiol-specific peroxiredoxin that reduces hydroperoxides to protect against oxidative damage (Fig. 1B). It is a known fungal allergen, is identical to peroxisomal membrane protein Pmp20 and, as a vaccine, has recently been demonstrated to induce cell-mediated protection against *Aspergillus* infection (Mabey et al., 2004; Diaz-Arevalo et al., 2011). Pmp20 characterisation in *Candida boidinii* suggested that it functioned to degrade ROS generated at peroxisomal membrane surface, such as lipid hydroperoxides (Horiguchi et al., 2001).

Interestingly, expression of several stress-induced proteins was decreased in the presence of exogenous gliotoxin including mycelial catalase and antioxidant protein, peroxiredoxin LsfA. Mycelial catalase (AFUA_3G02270) expression decreased nearly 42-fold in expression. Lessing et al. (2007) have shown that hydrogen peroxide exposure down-regulates expression of a distinct catalase (AFUA_2G00200) but did not mention AFUA_3G02270. Nonetheless, their observation is in accordance with our findings that oxidative stress conditions may, unexpectedly, suppress catalase levels. Recently, gliotoxin was identified as an antioxidant in mammalian cells, functioning as a peroxiredoxin in the thioredoxin redox system (Choi et al., 2007). Consequently, we speculate that exogenous gliotoxin may fulfil this biological function in *A. fumigatus*, resulting in diminished requirement for mycelial catalase. Peroxiredoxin LsfA (AFUA_4G08580) is a putative alkyl hyderoperoxide reductase/thiol specific antioxidant, and as such, may have several predicted functions such as antioxidant and oxidoreductase activities. In this case, it appears that gliotoxin exposure down-regulates, LsfA levels (approximately seven-fold), while H_2O_2 presence induces expression (Lessing et al., 2007). Overall, the differential regulation of 27 proteins (including one protein of unknown function, AFUA_5G14680) in response to exogenous gliotoxin suggests a hitherto unknown complex interaction between gliotoxin and *A. fumigatus.*

Increased expression (2.5–3.5-fold) of GliT (gliotoxin oxidoreductase; AFUA_6G09740), a component of the gliotoxin biosynthetic cluster was previously reported by us (Schrettl et al., 2010). Interestingly, Schrettl et al. (2008) also observed independent regulation of *gliT* during comparative transcriptomics study of *A. fumigatus* wild-type and a mutant deficient in control of siderophore biosynthesis (Δ*sreA*). Moreover, Bruns et al. (2010) detected increased *A. fumigatus* GliT expression during biofilm formation using a proteomic strategy, along with the detection of additional *gli* genes by transcriptomic analyses. Schrettl et al. (2010) suggested that gliotoxin addition to fungal cultures resulted in GSH depletion, and the data presented herein (Fig. 2B) confirm

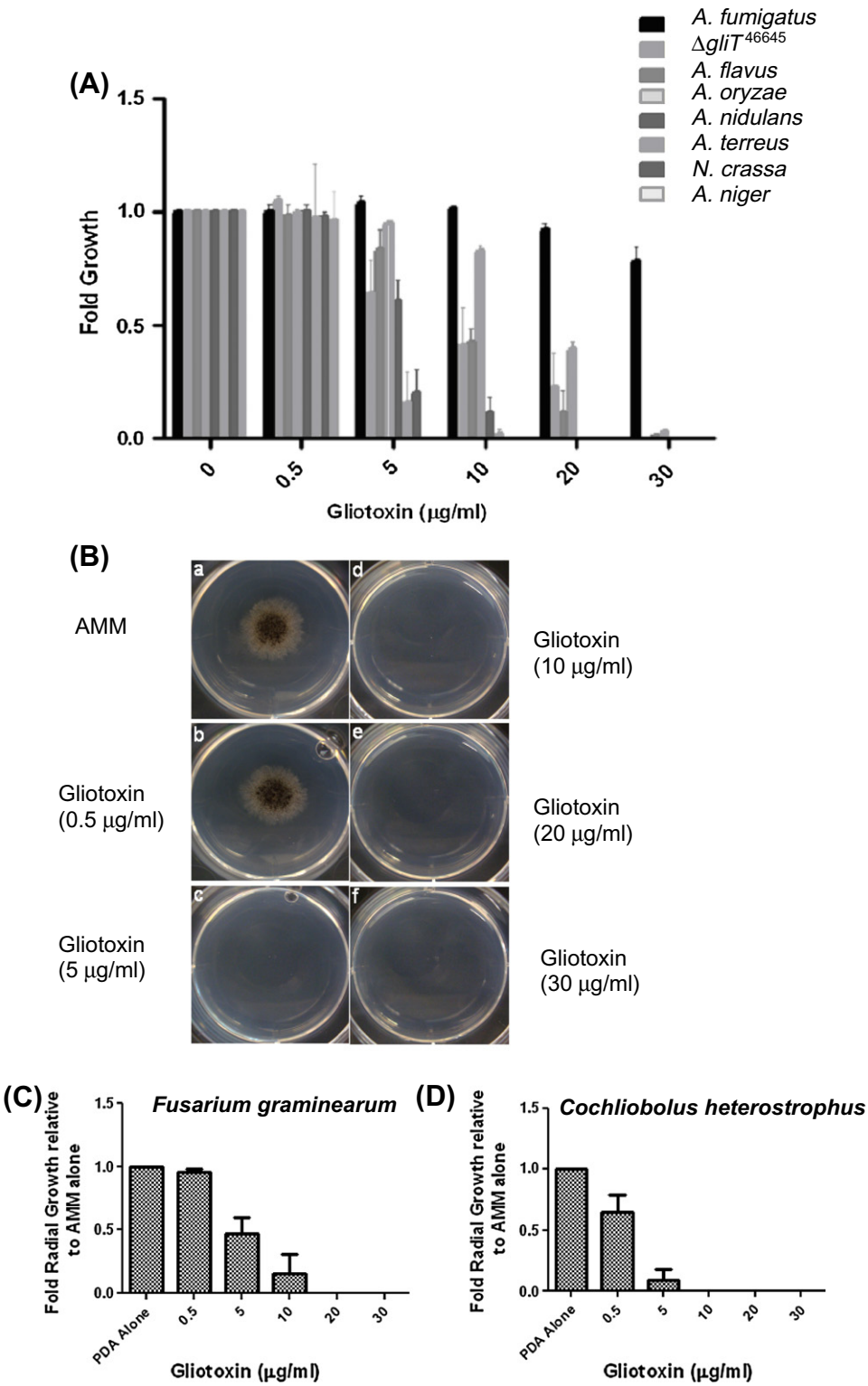

Fig. 4. (A) Sensitivity of a range of biomedically and commercially-important fungal strains to gliotoxin. (B) Visualisation of *A. niger* sensitivity to gliotoxin (0–30 µg/ml). (C and D) Growth of both *C. heterostrophus* and *F. graminearum* is significantly inhibited by exogenous gliotoxin ($p < 0.001$). These data indicate that all of the above strains may be suitable for use with *gliT*/gliotoxin selection marker system for both gene deletion studies and heterologous protein expression in fungi.

this prediction. However, we find that deletion of *gliT* results in significant elevation of intracellular GSH, with concomitant resistance to diamide, compared to *A. fumigatus* wild-type. This observation suggests that elevated intracellular GSH may actually potentiate the action of gliotoxin on *A. fumigatus* Δ*gliT*, yet simultaneously confer increased resistance towards the GSH-scavanging oxidant, diamide. Interestingly, *A. fumigatus* Δ*gliT* did not exhibit increased sensitivity to H_2O_2 (Schrettl et al., 2010), which suggests distinct patterns of redox stress in *A. fumigatus*. In this regard, Lessing et al. (2007) and Qiao et al. (2008) observed significantly increased sensitivity against H_2O_2 and menadione, but not against diamide, in *A. fumigatus* Δ*yap1*.

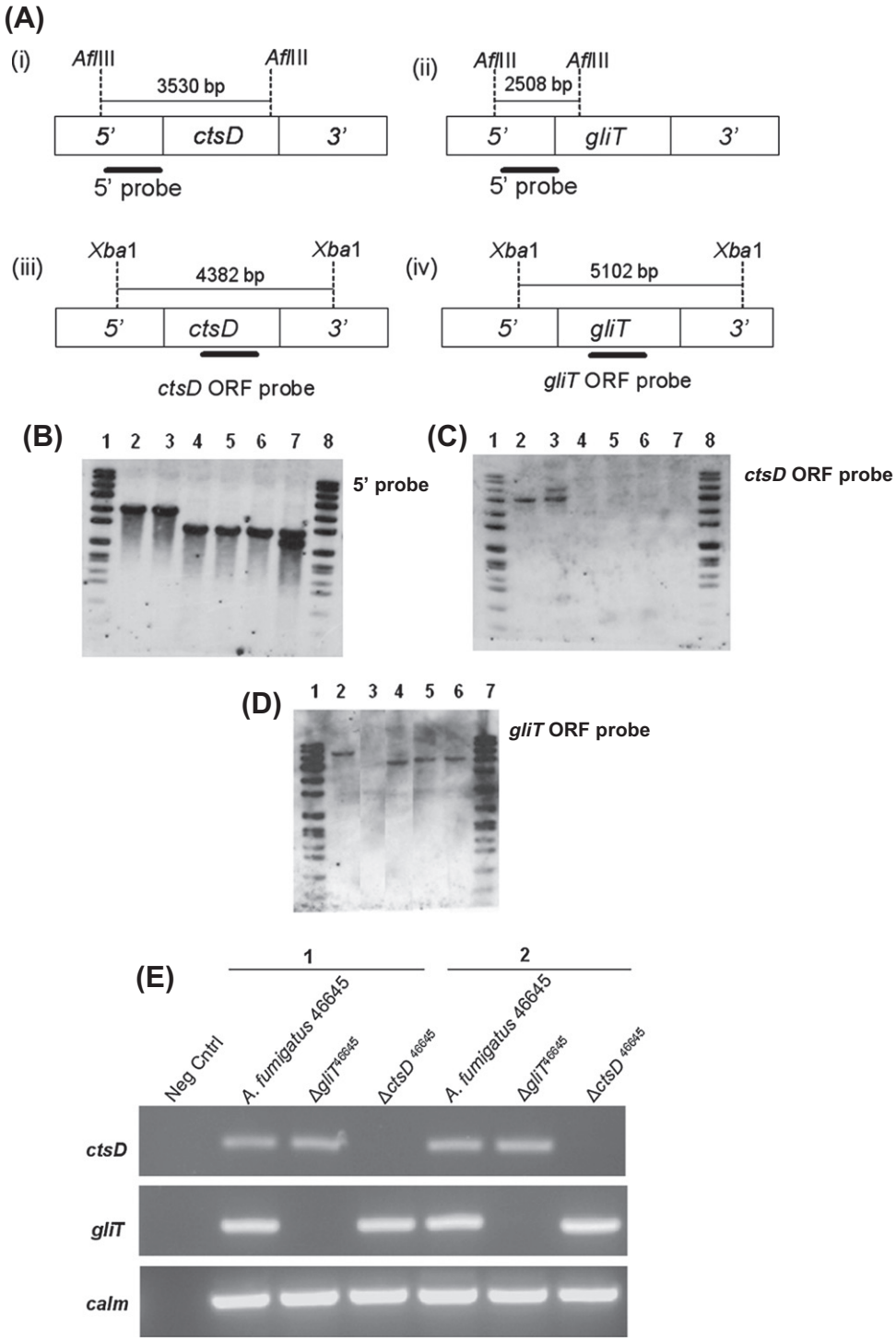

Fig. 5. Disruption of a *ctsD* in *A. fumigatus* Δ*gliT*^{46645} using *gliT* re-introduction at *ctsD* locus, followed by transformant selection on gliotoxin. (A) Schematic diagram showing probe hybridisation regions and predicted restriction fragment sizes. Southern blot analysis following: (B) *Afl*III digestion and 5′ *ctsD* detection probe. Expected bands: *A. fumigatus* ATCC46645 or Δ*gliT*: 3530 bp, Δ*ctsD*::*gliT*: 2508 bp. Appearance of a band at 2508 bp indicates disruption of *ctsD*. (C) *Xba*I digestion and *ctsD* coding sequence probe. Expected bands: *A. fumigatus* ATCC46645 or Δ*gliT*: 4832 bp Δ*ctsD*::*gliT*: no band. Absence of band at 4832 bp in lanes 4–7 indicates *ctsD* coding sequence is absent. D. *Xba*I digestion and *gliT* coding sequence probe. Expected bands: *A. fumigatus* ATCC46645 or Δ*ctsD*::*gliT*: 6403 or 5102 bp, respectively, Δ*gliT*: no band. *gliT* coding sequence re-introduced in Δ*gliT* at *ctsD* locus, as evidenced by appearance of band at 5102 bp. Key: 1. Markers. 2. *A. fumigatus* ATCC46645 3. *A. fumigatus* Δ*gliT*^{46645} 4. *A. fumigatus* Δ*ctsD*::*gliT*^{c} 5. *A. fumigatus* Δ*ctsD*::*gliT*^{c} 6. *A. fumigatus* Δ*ctsD*:*gliT*^{c} 7. *A. fumigatus* Δ*ctsD*::*gliT*^{c}. 8. Markers. E. Expression analysis, using duplicate cultures, of *gliT* and *ctsD*, respectively, using RT-PCR. Top panel: *ctsD* expression is absent in Δ*ctsD*. Middle panel: *gliT* expression is restored in strain Δ*ctsD*:*gliT*^{c}. Lower panel: RNA loading control.

Gliotoxin (8 μg/ml) also adversely affects *S. cerevisiae* whereby Δ*sod1* and Δ*yap1* growth, respectively, is inhibited to a greater extent than BY4741 (wild-type). This increased sensitivity of *S. cerevisiae* Δ*sod1* is in accordance with our observation that gliotoxin up-regulates Cu, Zn SOD expression in *A. fumigatus* and predicts superoxide production is consequent to gliotoxin addition in both fungal species. Moreover, the increased sensitivity to gliotoxin in *S. cerevisiae* Δ*yap1* implies that this gene is also essential to protect against gliotoxin-induced ROS. These data further the observations of Chamilos et al. (2008) regarding the effects of gliotoxin on yeast

mutants and future studies will explore the effects of gliotoxin on *A. fumigatus* Δ*yap1*. Our observation that *S. cerevisiae* Δ*gsh1*, severely deficient in GSH (Lee et al., 2001), exhibits greater resistance to gliotoxin than wild-type – and that complementation with *GSH1* restores wild-type levels of gliotoxin sensitivity - further underpins our proposal that intracellular GSH levels play an important role in mediating gliotoxin cytotoxicity in fungi as has been noted in animals cells by Bernardo et al. (2003).

Gliotoxin was originally investigated as an antifungal antibiotic (Johnson et al., 1943). Later, Losada et al. (2009) investigated the presence of antifungal agents produced by *Aspergillus spp* and detected gliotoxin in fungal co-culture extracts by LC-MS analysis. Yet, the dramatic sensitivity to gliotoxin (10 μg/ml; 30 μM) evident in the present work, whereby growth of three fungal species (*A. niger*, *N. crassa* and *C. heterostrophus*) was completed inhibited, was unexpected. Notably, all three strains lack the *gli* cluster and do not possess a *gliT* ortholog which, at least in part, explains this dramatic gliotoxin sensitivity compared to fungi which possess the cluster with multiple copies of *gliT* (*A. flavus*) (Patron et al. 2007; Fox and Howlett, 2008). Coleman et al. (2011) noted the sensitivity of *Candida albicans* and *Cryptococcus neoformans* to gliotoxin and *A. fumigatus* culture supernatants (containing gliotoxin), and speculate about the role of gliotoxin as a competitive factor during host colonisation by multiple fungal species. However, an alternative hypothesis could be that gliotoxin secretion represents a component of the defensive armoury of *A. fumigatus* against host oxidants, and that the antifungal activity is either accidental, or of more relevance in external environments to facilitate niche competition. Interestingly, Bruns et al. (2010) noted elevated levels of gliotoxin in *A. fumigatus* biofilms, compared to culture supernatants, and note that this may represent an immunomodulatory strategy (Nishida et al., 2005) on behalf of the organism. However, a more specific interpretation is that a localised gliotoxin "corona" acts externally to dissipate host ROS. If this hypothesis is true, then *A. fumigatus* would have to be able to withstand high local gliotoxin concentrations, which can, in part, explain the protective proteomic remodelling observed herein in response to exogenous gliotoxin addition.

Gliotoxin sensitivity offers the possibility of using *gliT* as a dominant selection marker for fungal transformation. Consequently, the successful deletion of *ctsD* (Vickers et al., 2007) from *A. fumigatus* Δ*gliT*, using *gliT* reconstitution and selection on gliotoxin proves this concept- at least in *A. fumigatus*. Further refinements of this system, which involves use of a fungal-specific gene (*gliT*), will involve use of self-excision mechanisms (Hartmann et al., 2010) to release *ptrA* originally used for *gliT* deletion (Schrettl et al. 2010) and investigation of the system in commercially important strains (e.g., *Trichoderma reesei*; Steiger et al., 2011).

5. Conclusion

We have demonstrated that gliotoxin addition perturbs the proteome of *A. fumigatus* and induces *de novo* Cu, Zn SOD expression. Combined with observations of significantly elevated GSH levels in *A. fumigatus* Δ*gliT* and the effects of gliotoxin on *S. cerevisiae*, we conclude that gliotoxin induces redox stress in fungi, at least in part mediated by superoxide anions and elevated GSH levels. While the antifungal effects of gliotoxin are clear, our results are also in accordance with a complex self-protection system, operative in *A. fumigatus*. Based on our observations, along with those of Schrettl et al. (2010), Watanabe et al. (2004), Bruns et al. (2010) and Coleman et al. (2011), we now hypothesise that this system may be necessary to defend against high, localised gliotoxin levels (Bruns et al., 2010), present during host colonisation- one function of which may be external dismutation of host ROS. Finally, we demonstrate extreme gliotoxin sensitivity in a range of fungi, the utility of *gliT*/gliotoxin for targeted gene deletion studies in *A. fumigatus*, and suggest that gliotoxin sensitivity assays should be incorporated into future functional genomic and proteomic studies involving fungi.

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