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Quantitative proteomics reveals the mechanism and consequence of gliotoxin-mediated dysregulation of the methionine cycle in *Aspergillus niger*

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ABSTRACT

Gliotoxin (GT) is a redox-active metabolite, produced by *Aspergillus fumigatus*, which inhibits the growth of other fungi. Here we demonstrate how *Aspergillus niger* responds to GT exposure. Quantitative proteomics revealed that GT dysregulated the abundance of 378 proteins including those involved in methionine metabolism and induced de novo abundance of two S-adenosylmethionine (SAM)-dependent methyltransferases. Increased abundance of enzymes *S*-adenosylhomocysteinase ($p = 0.0018$) required for homocysteine generation from *S*-adenosylhomocysteine (SAH), and spermidine synthase ($p = 0.0068$), involved in the recycling of Met, was observed. Analysis of Met-related metabolites revealed significant increases in the levels of Met and adenosine, in correlation with proteomic data. Methyltransferase MT-II is responsible for bisthiobis(methylthio)gliotoxin (BmGT) formation, deletion of MT-II abolished BmGT formation and led to increased GT sensitivity in *A. niger*. Proteomic analysis also revealed that GT exposure also significantly ($p < 0.05$) increased hydrolytic enzyme abundance, including glycoside hydrolases ($n = 22$) and peptidases ($n = 16$). We reveal that in an attempt to protect against the detrimental affects of GT, methyltransferase-mediated GT thiomethylation alters cellular pathways involving Met and SAM, with consequential dysregulation of hydrolytic enzyme abundance in *A. niger*. Thus, it provides new opportunities to exploit the response of GT-naïve fungi to GT.

1. Introduction

Gliotoxin (GT) is a redox-active non-ribosomal peptide produced by several *Aspergillus spp.* that has been extensively studied because of its cytotoxic and immunosuppressive effects on mammalian cells [1,2]. Model eukaryotic fungal organisms (e.g., *Saccharomyces cerevisiae*) have been used to screen genes associated with GT resistance or sensitivity as a model system for the impact of GT on animal cells [3], however, little is known about the effect that this toxin has on other fungi. The production of GT within the *Aspergillus* genus is inconsistent, for example, pathogenic fungi like *A. fumigatus* can produce GT while *A. niger* and *Aspergillus nidulans* lack the *gli* cluster responsible for the biosynthesis of the toxin [4]. It has been revealed that the secretome of *A. fumigatus* can inhibit the growth of *Candida albicans* and *Cryptococccus neoformans* through the production of GT [5]. The anti-fungal properties of GT have also been observed in *A. niger* [6], although the mechanism of this growth inhibition is unclear.

A self-protection mechanism, based on the GT oxidoreductase, GliT, and a membrane–localized MFS transporter, GliA, appears to have evolved in GT-producing, but not GT naïve fungi [7–10]. This system functions to oxidize a dithiol GT biosynthetic intermediate and secrete GT from the producing species, thereby attenuating the intracellular toxicity of the reduced form of the dithiol-containing compound. Moreover, transformation of *S. cerevisiae* with either *gliT* or *gliA* confers increased resistance to GT [7,9]. Recent work has revealed that certain *Aspergillus* spp. can also enzymatically methylate the thiols of reduced GT (rGT) to form bisthio*bis*(methylthio)gliotoxin (BmGT), and this reaction is catalysed by GT bisthiomethyltransferase A (GtmA) [11], termed thiol thiomethyltransferase (TtmA) in [12]. Unexpectedly, the deletion of *gtmA* from *A. fumigatus* does not seem to confer increased sensitivity to exogenous GT, despite abrogation of dithiol presence or prevention of redox cycling, and instead leads to an over-production of exogenous GT. In effect, BmGT formation primarily seems to serve as a negative regulatory mechanism to effect attenuation of GT biosynthesis in *A. fumigatus* [11]. Puzzlingly, although orthologs of GtmA are detected in certain GT-naïve fungi, including *A. niger* [11], the functionality of the encoded enzymes remains obscure, although BmGT formation has been demonstrated in *A. nidulans* upon exposure to exogenous GT [12]. MacKenzie et al. [13] demonstrated that

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dithiothreitol (DTT; 20 mM) significantly induced the increased expression of two methyltransferases (termed MT-I and MT-II) in *A. niger* and speculated as to their functionality in the unfolded protein response (UPR), although no definitive information was forthcoming on the biological function of either gene. Interestingly, we and others have noted that MT-II is an ortholog of GtmA (AFUA_2G11120) [11,12].

A. niger is a filamentous fungus widely used as a host for the production of native and heterologous proteins, and multiple enzymes with biotechnological uses have been identified in, and purified from, *A. niger* [14,15]. Indeed, the availability of the genome sequence of this highly useful biological cell factory has been available since 2007 which has facilitated its further development as a tractable system for recombinant protein expression [16]. However, protein overexpression in *A. niger* can be limited by redox stress and the UPR [17], and we speculated if revelation of the mechanism of GT sensitivity in *A. niger* could provide new insight into factors which negatively affect [18] protein expression. Studies of the transcriptional and translational response to stress agents (DTT and tunicamycin) in *A. niger* have been carried out in order to elucidate ways of overcoming this barrier to successful protein expression [13,19].

Herein, we investigate *A. niger* as a model to explore GT cytotoxicity. Label-free quantitative proteomic analysis was performed, followed by detailed dissection of selected aspects of the organismal response. We reveal that exogenous GT dysregulates the methionine cycle and specifically induces increased abundance of a number of methyltransferases, including MT-II. We demonstrate that *MT-II* deletion results in acquisition of a GT-sensitive phenotype and propose that MT-II is a component of the defence mechanism *A. niger* operates against GT. Furthermore, exogenous GT also positively dysregulates hydrolytic enzyme abundance, which we suspect plays a role in scavenging nutrients.

2. Methods

2.1. Plate assays

Conidia (5×10^3), harvested aseptically from one-week old Minimal Media plates, were used for phenotypic assays. Plates were incubated at 30 °C. Colony diameters were measured periodically and statistical analysis was carried out using one-way ANOVA.

2.2. Label-free quantitative (LFQ) proteomic analysis of A. niger CBS 513.88 exposed to GT

A. niger CBS 513.88 ($n = 4$ biological replicates per condition) was cultured in Potato Dextrose Broth (PDB) media for 45 h followed by GT (2.5 μg/ml, final) or methanol addition, respectively, for 3 h and 6 h. Mycelial lysates (500 mg) were prepared in lysis buffer (900 μl) (100 mM Tris–HCl, 50 mM NaCl, 20 mM EDTA, 10% (v/v) Glycerol, 30 mM DTT, 1 mM PMSF, 1 μg/ml pepstatin A, pH 7.5; lysis buffer A) with grinding, sonication and bead-beating and clarified using centrifugation at 12,000 ×*g* for 10 min. Resultant protein lysates (2 mg/ml; 200 μl) were precipitated using TCA/acetone and re-suspended in 100 mM Tris–HCl, 6 M urea, 2 M thiourea, pH 8 (60 μl). Samples (50 μg; 17 μl) were reduced and alkylated with DTT and IAA respectively [20]. Sequencing grade trypsin (1 μg/μl; 1.6 μl) combined with ProteaseMax (1% (w/v); 1 μl) was added. After desalting (C_{18} ZipTips (Millipore)), peptides were analysed via a Thermo Scientific Q-Exactive mass spectrometer coupled to a Dionex RSLCnano. LC gradients ran from 2–40% B (A: 0.1% (v/v) formic acid, B; 80% (v/v) ACN, 0.1% (v/v formic acid) over 2 h, and data was collected using a Top15 method for MS/MS scans. Comparative proteome abundance was performed using MaxQuant software (Version 1.3.0.5) [21], with Andromeda used for database searching and Perseus (Version 1.4.1.3) used to organize the data. All other conditions were as described Owens et al. [10]. Quantitative analysis was performed using a t-test to compare pairs of samples, and proteins with significant change in abundance (p value <0.05; fold change ≥2) were found in at least 3 replicates of a particular sample, but undetectable in the comparitor sample. Enrichment analysis was carried out on proteins exhibiting altered abundance by FunCat [22] category. A p value cut-off of 0.05 was used. Additionally, qualitative analysis was performed using Proteome Discoverer Software (Version 1.4.0.288, Thermo Scientific).

2.3. Detection of A. niger intracellular S-adenosylmethionine, S-adenosylhomocysteine and adenosine

PDB media was inoculated with 10^7/ml conidia (*A. niger* CBS 513.88), in triplicate, and incubated at 30 °C, shaking 200 rpm, for 45 h. GT (2.5 μg/ml, final) or methanol solvent control was added and the cultures were incubated for a further 3 h before mycelia were harvested and snap frozen in liquid N_2. *S*-adenosylmethionine (SAM), *S*-adenosylhomocysteine (SAH) and Adenosine (Ado) were extracted as described by [23], with modifications. Mycelia were ground under liquid N_2 using a pestle and mortar. 0.1 M HCl (250 μl) was added to mycelia (100 mg) and incubated on ice for 1 h with regular vortexing. Following centrifugation at 13,000 ×*g*, protein was removed from the supernatant by TCA precipitation. Samples were diluted in 0.1% (v/v) formic acid and analysed by LC–MS/MS using a porous graphitized carbon (PGC) chip on a 6340 Ion-trap LC Mass Spectrometer using electrospray ionization (Agilent Technologies) [10]. SAM levels were determined as Relative Quantification (RQ) by LC–MS analysis. SAH levels were determined as Absolute Quantification (AQ) by RP–HPLC by specimen comparison to a calibration curve (6.25–400 μg/ml SAH). Statistical analyses were carried out using Student's t-test.

2.4. RP–HPLC detection of S-adenosylhomocysteinase activity

To analyse SAH enzymatic breakdown, *A. niger* CBS 513.88 was grown ($n = 3$ biological replicates) at 30 °C for 48 h in PDB and mycelia extracted as per SAH determination assay. SAH (50 μg/ml final concentration) was spiked into the *A. niger* lysates and samples were analysed using RP–HPLC; (C_{18} column, Agilent Zorbax Eclipse XDB-C18) (Agilent 1200 System) after 0, 35, 70 and 105 min post-spiking. Gradient elution was performed with Solvent A consisting in 25 mM NaH_2PO_4 and 2.3 mM octane sulfonic acid, pH 4.5, in HPLC grade water (Sigma-Aldrich) and Solvent B consisting of 100% ACN [24,25]. All cultures were grown up in triplicate and each one was compared to a standard curve (6.25–400 μg/ml SAH).

2.5. Detection and quantification of methionine

Intracellular methionine (Met) was measured using the method of Sun. et al. [26], with modifications. *A. niger* CBS 513.88 ($n = 3$ biological replicates) was cultured for 45 h in PDB at 30 °C prior to GT addition (2.5 μg/ml, final) and methanol solvent control for 3 h. Mycelia were harvested through miracloth and snap frozen in liquid N_2. Mycelia (500 mg) in lysis buffer A (900 μl) were sonicated, bead-beaten and clarified using centrifugation. Protein concentrations were quantified by Bradford Assay and brought to the same concentration. *O*-phthaldialdehyde (OPA) (50 μl) was added to *A. niger* lysates (10 μl; 1/5 in 50 mM sodium phosphate pH 7.6) to effect amino acid derivatization. All samples were incubated for 90 s at room temperature and immediately loaded (20 μl) on a RP-HPLC C_{18} column (Agilent Zorbax Eclipse XDB-C18). Gradient elution was performed with Solvent A consisting of 0.1% (v/v) TFA in 99.9% HPLC grade water (Sigma-Aldrich) and Solvent B consisting of 0.1% (v/v) TFA in 99.9% ACN (Sigma-Aldrich). Quantification was enabled using commercially available Met obtained from Sigma-Aldrich. Statistical analyses were carried out using Student's t-test.

2.6. Detection of GT and BmGT in Aspergillus spp. supernatants

A. niger strains ($n = 3$ biological replicates) and *A. nidulans* FGSC4 ($n = 3$ biological replicates) were grown at 30 °C and 37 °C respectively. Freshly harvested conidia (10^7/ml) were inoculated in PDB and transformation media with shaking at 200 rpm for 45 h. At 45 h, GT (2.5 μg/ml, final) or methanol (solvent control) were added to the cultures, and 2 ml aliquots of supernatant were removed at 0, 30, 60, 120, 180 and 240 min. After chloroform extraction, all specimens were resuspended in methanol (20 μl) followed by LC–MS/MS and RP–HPLC analysis [11]. GT and BmGT levels were determined either by RQ or AQ. All strains used are indicated in Table S1.

2.7. GT thiomethyltransferase activity

A. niger CBS 513.88 mycelial lysates were obtained following 48 h growth in PDB media (45 h culture followed by supplementation with GT (2.5 μg/ml) (Induced) or methanol (Uninduced) for 3 h. Mycelia were snap-frozen and ground in liquid N_2, and lysed by sonication and bead-beating in lysis buffer A. Protein lysates were clarified by centrifugation prior to use in assays. Reaction mixtures were carried out essentially as described by [11] with modifications: Tris (2-carboxyethyl) phosphine (TCEP)-reduced GT (1 mg/ml; 2 μl), SAM (25 mg/ml in PBS, 3 μl), *A. niger* lysate (20 μl; 3 mg/ml protein) and PBS (75 μl). Negative controls used 20 μl lysis buffer instead of lysate. Reactions were incubated at 37 °C for 3 h and terminated by adjustment to 15% (v/v) TCA, vortexed briefly and incubated on ice for 30 min. Samples were centrifuged at 10,000 ×*g* for 10 min, 4 °C and supernatants were diluted 1/10 in 0.1% (v/v) formic acid and spin filtered prior to LC–MS/MS analysis (Agilent Ion Trap 6340) to detect thiomethylated GT derivatives [11].

2.8. MT-II expression and purification

The *A. niger MT-II* (AspGD ID: An02g03100) gene was PCR amplified from *A. niger* CBS 513.88 cDNA using appropriate primers (Table S2), the PCR product was digested with *Xho*I and *Hind*III and cloned into the pEX-N-GST PrecisionShuttle vector (OriGene) according to the manufacturer's instructions, resulting in pEX-N-GST-*MT-II*. One Shot® BL21 Star™ (DE3) chemically competent *Escherichia coli* carrying the pEX-N-GST-*MT-II* vector were grown in LB broth containing ampicillin (100 μg/ml) at 37 °C. Protein production was induced by Isopropyl β-D-1-thiogalactopyranoside (IPTG; 0.5 mM) before the culture was further incubated for 12 h at 25 °C. Cells were harvested and lysed (lysozyme (90 μg/ml) in the presence of inhibitors (1 μg/ml pepstatin and 1 mM PMSF) and insoluble material was removed by centrifugation (8000 ×*g*, 30 min, 4 °C). The soluble fraction, containing the recombinant MT-II-GST, was subjected to GSH-Agarose affinity chromatography using an AKTA Purifier-100. Protein purity was assessed by SDS-PAGE and Western blot analysis using IgG (anti-GST/HRP) antibody (Amersham Biosciences) (1/5000), and diaminobenzidine (DAB) detection.

2.9. Construction of deletion cassette and fungal transformation

In order to create a deletion cassette for *MT-II* deletion, 2.5 kb 5′-UTR (OFM24/25) and 3′-UTR (OFM26/27) flanking sequences of *MT-II* were amplified from *A. niger* genomic DNA. The *hph* cassette was amplified with OFM28/29 from pAN7-1. These three fragments were ligated into *Sma*I site of pUC19 by using an in-fusion HD cloning kit (Clontech), which led to pOFM8 (*hph*::*MT-II*Δ cassette). The 6.945 kb deletion cassette was amplified with nested primers OFM34/35 (Table S2), which was used for transformation and gene replacement event in an *A. niger* uridine auxotroph (N593; a kind gift from Dr. Arthur F.J. Ram, Leiden University, NL). *A. niger* CBS 513.88 proved refractory to transformation. Diagnostic PCRs were performed with OFM65/46 for in-locus cassette control, and OFM70/71 for *MT-II* ORF amplification.

A. niger N593 (10^6 spores/ml) was inoculated into 100 ml liquid transformation medium (TM) containing 2% Glucose (TM: (salt solution 70 mM $NaNO_3$, 7 mM KCl, 11.2 mM KH_2PO_4, 2 mM $MgSO_4$, trace elements 76 μM $ZnSO_4$, 178 μM H_3BO_3, 25 μM $MnCl_2$, 18 μM $FeSO_4$, 7 μM $CoCl_2$, 6.4 μM $CuSO_4$, 6.2 μM Na_2MoO_4, 174 μM EDTA, yeast extract 5 g/l, 2 g/l casamino acids) supplemented with 1 g/l uracil and 1 mM uridine and grown for 20 h at 30 °C. Transformation of fungal spores were performed according to common *Aspergillus* transformation protocols [27]. 2–4 μg linear deletion cassette was used for transformation of generated protoplasts. Protoplasts were plated on TM plates without yeast extract and casamino acids, containing 1.2 M sorbitol as osmotic stabilizer and hygromycin (200 μg/ml; InvivoGen) as selective antibiotic supplemented with uracil and uridine. The plates were incubated at 30 °C for 4–6 days. Primary transformants were streaked out on the same TM plates without sorbitol. Diagnostic PCRs were performed with OFM65/46 for in-locus cassette control, and OFM70/71 for *MT-II* ORF amplification. Subsequently, GT sensitivity and its impact on germination of *A. niger* Δ*MT-II*, compared to wild-type, was evaluated (Supplementary data). *MT-II* was also transformed into *S. cerevisiae*, essentially as described for *gliT* [7], followed by expression and GT sensitivity analysis, as well as evaluation of BmGT formation by RP–HPLC.

3. Results

3.1. GT exposure alters the abundance of hundreds of proteins in A. niger CBS 513.88

A. niger CBS 513.88 (wild-type) is unable to biosynthesise GT and is sensitive to exogenous GT in a concentration dependent manner (Fig. S1). Thus, it represents an alternative model organism to explore GT cytotoxicity and reveal new metabolic systems in filamentous fungi. LFQ proteomics was used to identify changes to the *A. niger* wild-type proteome subsequent to GT exposure for 3 h and 6 h. The times of exposure chosen were based on preliminary analyses (data not shown) which revealed that 3 h generated a greater proteomic response compared to earlier time points. 6 h was chosen in order to observe signs of recovery or enhanced sensitivity by GT in the fungi. Exposure to GT (3 h) resulted in significantly altered abundance ($p < 0.05$) of 378 proteins, compared to methanol-treated controls. Of these 378 proteins, 158 proteins exhibited increased abundance when *A. niger* was exposed to GT, while 220 proteins were less abundant (Tables S3 and S4). In contrast, when *A. niger* was exposed to exogenous GT for 6 h, 343 proteins were significantly altered in abundance ($p < 0.05$; increased: 187 proteins. decreased: 156 proteins) (Tables S5 and S6).

Functional analysis of the differentially abundant proteins was performed using the Functional Catalogue (FunCat) annotation tool [22]. Following *A. niger* exposure to exogenous GT for 3 h and 6 h, FunCat analysis generated 4 categories over-represented in the increased abundant protein sets at both time points. These included metabolism (3 h; $n = 74$, $p < 0.035$ and 6 h; $n = 99$, $p < 0.0003$), cell rescue, defence and virulence (3 h; $n = 25$, $p < 0.014$ and 6 h; $n = 34$, $p < 0.022$), interaction with the environment (3 h; $n = 16$, $p < 0.001$ and 6 h; $n = 23$, $p < 0.002$) and cell cycle and DNA processing (3 h; $n = 16$, $p < 0.034$ and 6 h; $n = 22$, $p < 0.046$). Categories enriched at 3 h included protein synthesis ($n = 17$, $p < 0.015$) and cellular transport, transport facilitation and transport routes ($n = 31$, $p < 0.016$), while categories enriched only at 6 h include transcription ($n = 18$, $p < 0.0003$) and regulation of metabolism and protein function ($n = 12$, $p < 0.047$) (Fig. 1A). However, functional classification of proteins with reduced abundance after GT exposure (3 h) were metabolism ($n = 104$, $p < 0.041$), energy ($n = 51$, $p < 8.72e^{-06}$) and cell type differentiation ($n = 17$, $p < 0.047$). Protein with binding function or cofactor

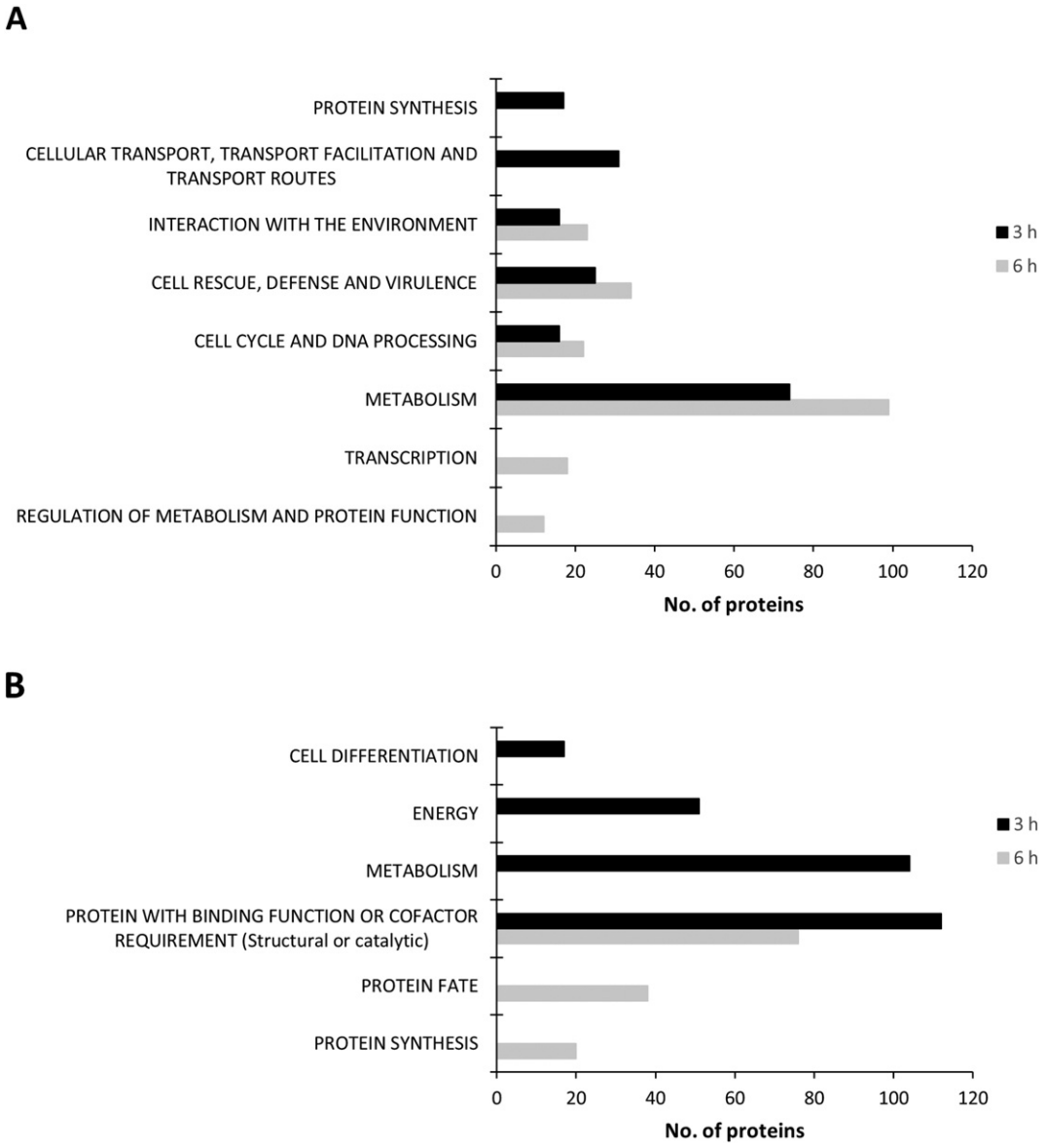

Fig. 1. GT exposure induces significant changes in protein abundance in *A. niger*. Functional classification into FunCat 1st level categories of significantly (A) protein families with increased abundance in *A. niger* CBS 513.88 exposed to GT compared to the MeOH control and (B) protein families with decreased abundance in *A. niger* CBS 513.88 exposed to GT compared to the MeOH control.

requirement (structural or catalytic) was enriched in both time points (3 h; $n = 112$, $p < 1.16e^{-05}$ and 6 h; $n = 76$, $p < 0.001$) and categories over-represented in the decreased abundance set after 6 h included protein fate ($n = 38$, $p < 0.016$) and protein synthesis ($n = 20$, $p < 0.001$) (Fig. 1B).

3.2. GT exposure increases the abundance of proteins involved in the methionine cycle

Dysregulation of enzymes involved in methionine and cysteine metabolism was observed in *A. niger* following exposure to exogenous GT for 3 h (Fig. 2), whereby five proteins exhibited reduced and three increased abundance. In contrast, 2 proteins decreased in abundance in *A. niger* in response to exogenous GT for 6 h and 2 proteins were evident with increased abundance (Table 1). *S*-adenosylhomocysteinase (SAHase), which catalyses the degradation of SAH to homocysteine (Hcy) and Ado, was increased in abundance by $\log_2$ 2.46-fold and 1.45-fold at 3 h and 6 h, respectively, whilst homoserine dehydrogenase was similarly abundant at both time points ($\log_2$1.96-fold at 3 h and $\log_2$1.89-fold at 6 h). Spermidine synthase, involved in methionine recycling and polyamine biosynthesis [28] was increased in abundance $\log_2$ 2.00-fold (at 3 h). Of the 5 proteins exhibiting decreased abundance in *A. niger* after 3 h GT exposure, 2 homologous aspartate aminotransferases (An16g05570 and An04g06380) and 2 homologous malate dehydrogenases (An07g02160 and An15g00070), which are part of the TCA cycle and the malate–aspartate shuttle [29] also appear to be involved in the generation of pyruvate from cysteine according to KEGG classification. Cysteine synthase, which catalyses the formation of cysteine from *O*-acetylserine, was not detectable after GT exposure (at 3 h). Two additional proteins showing reduced abundance after 6 h GT exposure were An04g06380 ($\log_2$1.90-fold) and An11g09510 ($\log_2$1.03-fold) which are involved in aspartate biosynthesis.

Furthermore, in addition to the dysregulation of the cysteine and methionine metabolism, two SAM-dependent methyltransferases (MTases), which utilize SAM as a methyl donor in trans-methylation reactions, showed de novo abundance in response to GT exposure (MT-I and MT-II at 3 h; MT-I and methyltransferase An18g03220 at 6 h)(Table 2). Abundance of an *N*-methyltransferase (An02g08430)

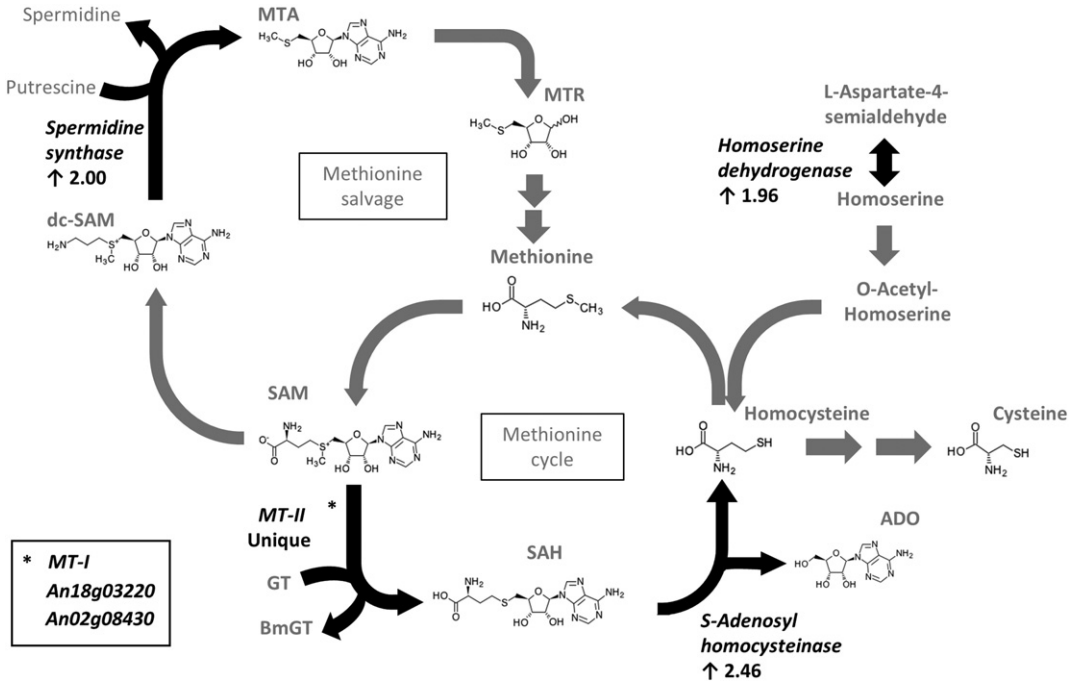

Fig. 2. GT exposure increases abundance of proteins involved in the methionine cycle. Enzymes in black are increased in abundance after GT addition (3 h) in *A. niger* CBS 513.88. Activity of MT-II, an ortholog of *A. fumigatus* GtmA, utilizes SAM to effect bis-thiomethylation of rGT to BmGT.

was also increased ($\log_2$1.44-fold at 3 h) after GT addition (Table 1). Given the unexpected detection of these MTases and their potential interaction with the methionine cycle, we analysed whether the levels of Met-related metabolites were affected. All together, these data demonstrate that the impact GT has in the methionine cycle and cysteine metabolism is greater at 3 h exposure compared to 6 h which suggests that over time, *A. niger* may be able to recover after GT exposure.

3.3. Met-related metabolite profiling

Determination of Met levels and associated metabolites in *A. niger* grown under identical conditions to those deployed for proteomic analyses (45 h growth + 3 h GT exposure) revealed significant alterations. Specifically, Met levels were significantly increased in *A. niger* after GT exposure ($p = 0.0132$) compared to methanol-treated controls (Fig. 3A). Conversely, SAM levels were significantly reduced ($p = 0.036$)(Fig. 3B), which suggests depletion of SAM by increased MTase activity consequent to GT exposure. In order to determine SAH levels, *A. niger* lysates in 0.1 M HCl were prepared according to Owens et al. [10]. Surprisingly, SAH was undetectable in *A. niger* acid lysates, even though it is acid-stable (Fig. 3C; [10]). Consequentially, we analysed whether or not SAH could be enzymatically degraded in *A. niger* acid lysates. A time and concentration-dependent disappearance of SAH was evident in *A. niger* acid lysates containing spiked SAH (initial conc. 50 μg/ml; Fig. 3D), indicating an enzyme-dependent process. Subsequent LC–MS/MS analysis of these *A. niger* acid lysates revealed the presence of SAHase (Table S7), which appears to explain the progressive reduction of added SAH and its absence in native, non-spiked, samples. A significant increase ($p = 0.013$) in Ado, a product of the SAHase-catalysed reaction (Fig. 2), was observed in GT-exposed samples

Table 1
Abundance changes of proteins involved in the cysteine and methionine metabolism in *A. niger* CBS 513.88 after exposure to gliotoxin (2.5 μg/ml) for 3 h and 6 h, respectively.

Protein description	$\log_2$ fold-change 3 h	$\log_2$ fold-change 6 h	EC number	Accession
Cysteine and methionine metabolism				
S-adenosylhomocysteinase	↑ 2.463	↑ 1.454	3.3.1.1	An08g01960
Ortholog(s) have spermidine synthase activity, role in pantothenate biosynthetic process, spermidine biosynthetic process and cytosol, extracellular region, nucleus localization	↑ 2.004	–	2.5.1.16	An08g06560
Homoserine dehydrogenase	↑ 1.969	↑ 1.899	1.1.1.3	An02g07430
Cysteine synthase	Absent	–	2.5.1.47	An02g10750
Aspartate transaminase; phenylalanine:alphaketoglutarate aminotransferase	↓ 2.707	–	2.6.1.1	An16g05570
Mitochondrial aspartate aminotransferase	↓ 2.506	↓ 1.906	2.6.1.1	An04g06380
Mitochondrial malate dehydrogenase	↓ 1.418	–	1.1.1.37	An07g02160
Malate dehydrogenase precursor; Malate dehydrogenase	↓ 1.321	–	1.1.1.37	An15g00070
Aspartic beta semi-aldehyde dehydrogenase, aspartate semialdehyde dehydrogenase	–	↓ 1.036	1.2.1.11	An11g09510
Sulphur metabolism				
Has domain(s) with predicted electron carrier activity, molybdenum ion binding, oxidoreductase activity and role in oxidation–reduction process	↑ 1.214		1.8.3.1	An08g08910
ATP sulfurylase	↓ 2.278	–	2.7.7.4	An11g09790
Ortholog(s) have electron transporter, transferring electrons from CoQH2-cytochrome c reductase complex and cytochrome c oxidase complex activity	–	↓ 1.515	–	An02g01830

Table 2
Abundance changes in SAM-dependent methyltransferases in *A. niger* CBS 513.88 after exposure to gliotoxin (2.5 μg/ml) for 3 h and 6 h, respectively.

Protein description	Log_2 fold-change 3 h	Log_2 fold-change 6 h	EC Number	Accession
Putative methyltransferase (MT-II)	Unique	–	–	An02g03100
Putative SAM-dependent methyltransferase (MT-I)	Unique	Unique	–	An08g10700
Ortholog(s) have double-stranded DNA 5′-3′ exodeoxyribonuclease activity, single-stranded DNA endodeoxyribonuclease activity, tRNA (m5U54) methyltransferase activity and role in double-strand break repair, tRNA modification	–	Unique	2.1.1.35	An18g03220
Ortholog(s) have histone-arginine N-methyltransferase activity, ribosome binding activity and role in peptidyl-arginine methylation, to asymmetrical-dimethyl arginine, ribosome biogenesis	↑ 1.448	–	2.1.1.—	An02g08430
Aminomethyltransferase	↓ 1.028	–		An08g03070

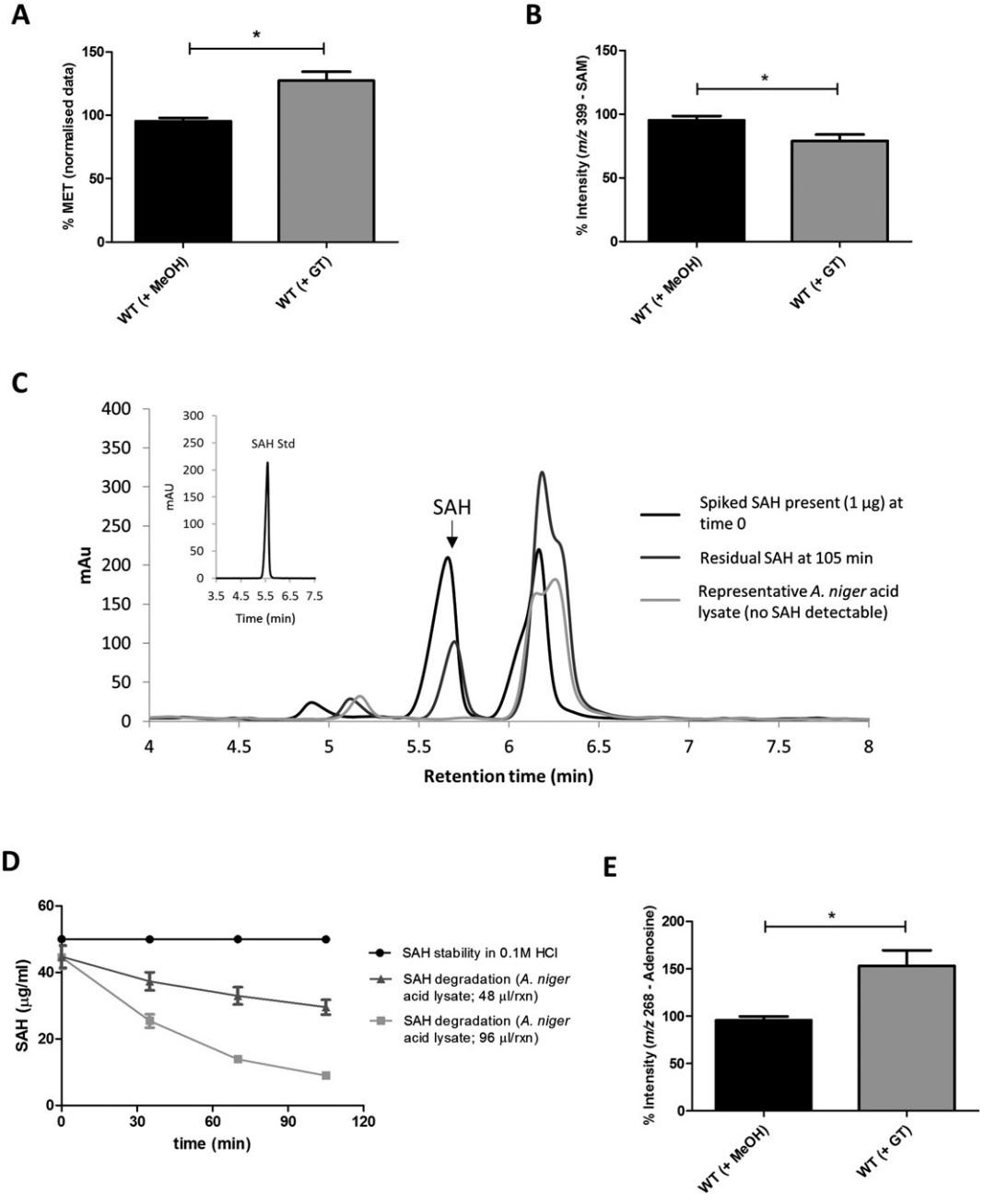

Fig. 3. Levels of methionine related metabolites in *A. niger* CBS 513.88 exposed to GT (2.5 μg/ml) for 3 h compared to methanol control. (A) Methionine levels were significantly increased in *A. niger* exposed to GT ($^*p = 0.0132$). (B) Significant reduction in SAM levels ($^*p = 0.036$). (C) Overlaid HPLC chromatograms of *A. niger* HCl lysates analysed at time 0 min (black) and 105 min (dark grey) after SAH spiking and *A. niger* HCl lysate with no SAH addition (clear grey). Spiked SAH eluted at $R_T = 5.66$ min (black arrow). There was no SAH detection in *A. niger* lysates. (D) SAH degradation activity was found in *A. niger* HCl lysates whereby spiked SAH disappeared in a time- and concentration-dependent fashion. (E) Ado levels were significantly increased in *A. niger* lysates after GT exposure ($^*p = 0.013$).

compared to methanol-treated controls (Fig. 3E), which infers that SAH undergoes rapid hydrolysis in *A. niger*.

3.4. In vivo and in vitro methylation of exogenous GT by A. niger

Scrutiny of MTases (Table 2) showing increased abundance under GT exposure (3 h and 6 h) revealed MT-I and MT-II, which were also found to undergo increased expression following DTT exposure in an *A. niger* subtraction library [13]. A BLASTP search confirmed that the closest *A. fumigatus* homologue of the *A. niger* MT-II is encoded by AFUA_2G11120, recently characterized as GtmA, which is a SAM-dependent GT bis-thiomethyltransferase, which converts dithiol or reduced GT (rGT) to BmGT [11]. Based on this observation, and in order to confirm if *A. niger* CBS513.88 is capable of thiomethylating GT, GT (final conc. 2.5 μg/ml) was added to *A. niger* liquid cultures over a 4 h period. We observed uptake of GT by *A. niger* and conversion to BmGT, which is secreted to the culture medium over a 4 h time-course (Fig. 4A). An identical in vivo bis-thiomethylation process is observed in *A. nidulans* FGSC4 exposed to exogenous GT (2.5 μg/ml) (Fig. S2) over a 4 h period. Interestingly, *A. nidulans* is also sensitive to exogenous GT [6], and is devoid of the *gli* cluster responsible for GT biosynthesis [4]. This observation shows that GT-naïve *Aspergillus spp.* are able to bis-thiomethylate exogenous GT. Using an LC–MS/MS assay for the determination of GT thiomethyl derivatives, significant ($p < 0.0006$) monodethiomono(methylthio)GT (MmGT; m/z 343) was detectable only in reactions catalysed by protein lysates derived from *A. niger* exposed to exogenous GT (2.5 μg/ml for 3 h) (Fig. 4B). When un-exposed control lysates were used (protein lysates derived from *A. niger* liquid cultures exposed to methanol only), no thiomethylation of GT was detectable (Fig. 4C). These data strongly suggested that a specialized thiol methyltransferase, present only after GT exposure, possibly MT-II, mediates the observed bioactivity.

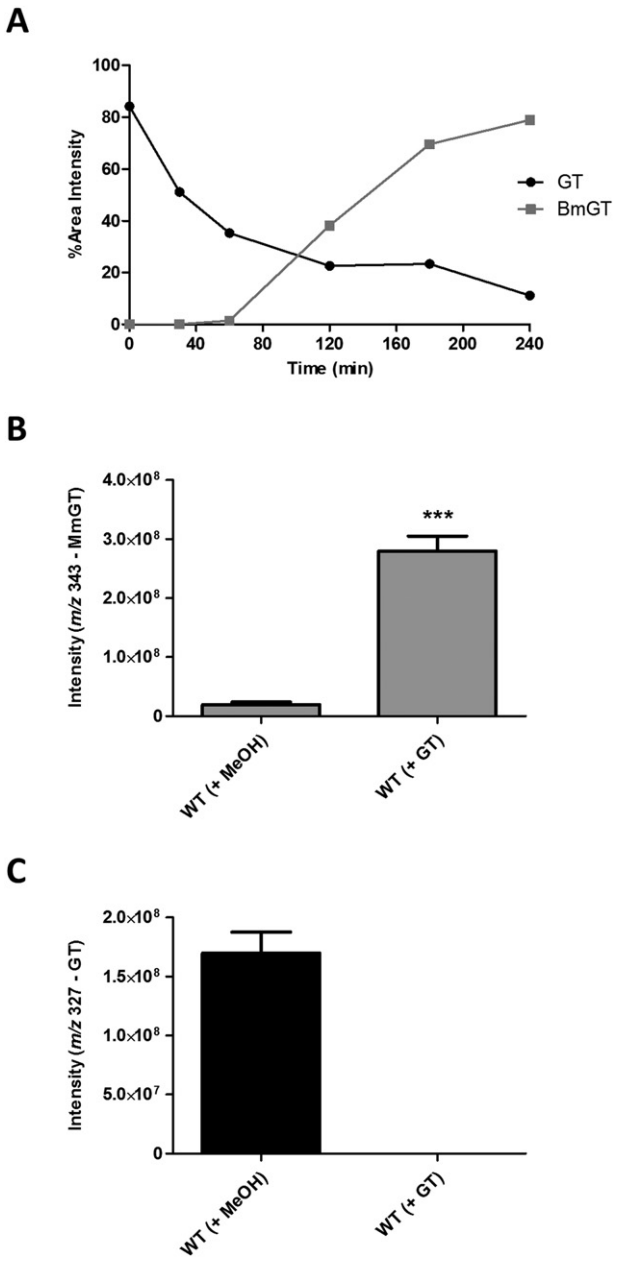

Fig. 4. (A) GT (2.5 μg/ml) added to *A. niger* CBS 513.88 cultures is converted to BmGT over 4 h. (B) *A. niger* protein lysates obtained only from GT-induced cultures can effect GT thiomethylation. (C) *A. niger* protein lysates obtained from un-induced cultures are unable to thiomethylate GT.

3.5. Expression, purification and biochemical analysis of recombinant MT-II

Quantitative reverse transcriptase PCR (qRT-PCR) analysis of *A. niger MT-II* expression confirmed the up-regulation ($p < 0.0001$) in *A. niger* exposed to exogenous GT for 3 h (Fig. S3). In agreement with the observations in [13], *MT-II* was also induced by DTT, but by almost 1000 times less on an equivalent molar basis of additive (GT; 7.7 μM versus DTT (10 mM)) (Fig. S3). After *A. niger* CBS 513.88 *MT-II* cDNA sequence confirmation (Fig. S3), recombinant expression of MT-II as a soluble GST fusion protein in *E. coli* (Fig. 5A), along with LC–MS identity confirmation, led to the assessment of activity of MT-II. Fig. 5B depicts how, in vitro, recombinant MT-II progressively thiomethylates rGT, using SAM as the methyl donor, through the sequential formation of MmGT and then BmGT. Indeed BmGT levels were highest after 3 h reaction time, compared to 1 h (43%) and 2 h (73%) (Fig. S4). In order to further characterize recombinant MT-II, the capacity of this enzyme to methylate GT to MmGT and BmGT was investigated (enzyme concentration, reaction temperature, pH and inhibitors). The optimal reaction temperature and pH was 37 °C (Fig. S4) and pH 7.0, respectively (Fig. S4). Fig. 5C shows that GT bis-thiomethylation is dependent on MT-II concentration (120 pmol/reaction). MmGT and BmGT levels were observed to decrease and increase, respectively, and simultaneously, as the concentration of MT-II increased. This data strongly suggests that rGT methylation proceeds in a sequential manner, involving MmGT release and re-binding to MT-II. Finally, addition of SAH to the reaction mixtures inhibited BmGT formation in a concentration dependent fashion, whereby a 2 fold molar excess of SAH over SAM leads to a 74% decrease compared to SAH absence (Fig. 5D).

3.6. A. niger ΔMT-II acquires a GT hypersensitivity phenotype

Deletion of *MT-II* in *A. niger* was undertaken in order to assess functionality of the recombinant MT-II (Fig. 6A). After confirming the absence of *MT-II* by diagnostic PCR (Fig. 6B) and the inability of the mutant to thiomethylate exogenously added GT compared to wild-type (Fig. 6C), phenotypic assays were carried out. *A. niger ΔMT-II* growth was significantly ($p < 0.0001$) reduced under GT addition compared to wild-type (Fig. 6D, E), which strongly infers that MT-II has a protective function against exogenous GT. Data in Figs. S5 and S6 reveal growth inhibition between conidial inocula 10^2–10^5, and that GT significantly inhibited the germination rate of *A. niger ΔMT-II* ($p < 0.01$). Transformation of *S. cerevisiae* BY4741, which lacks a *gtmA* ortholog, with *MT-II*/*gtmA* resulted in *gtmA* expression, acquisition of resistance to exogenous GT and an ability to convert added GT to BmGT (Figs. S7 and S8).

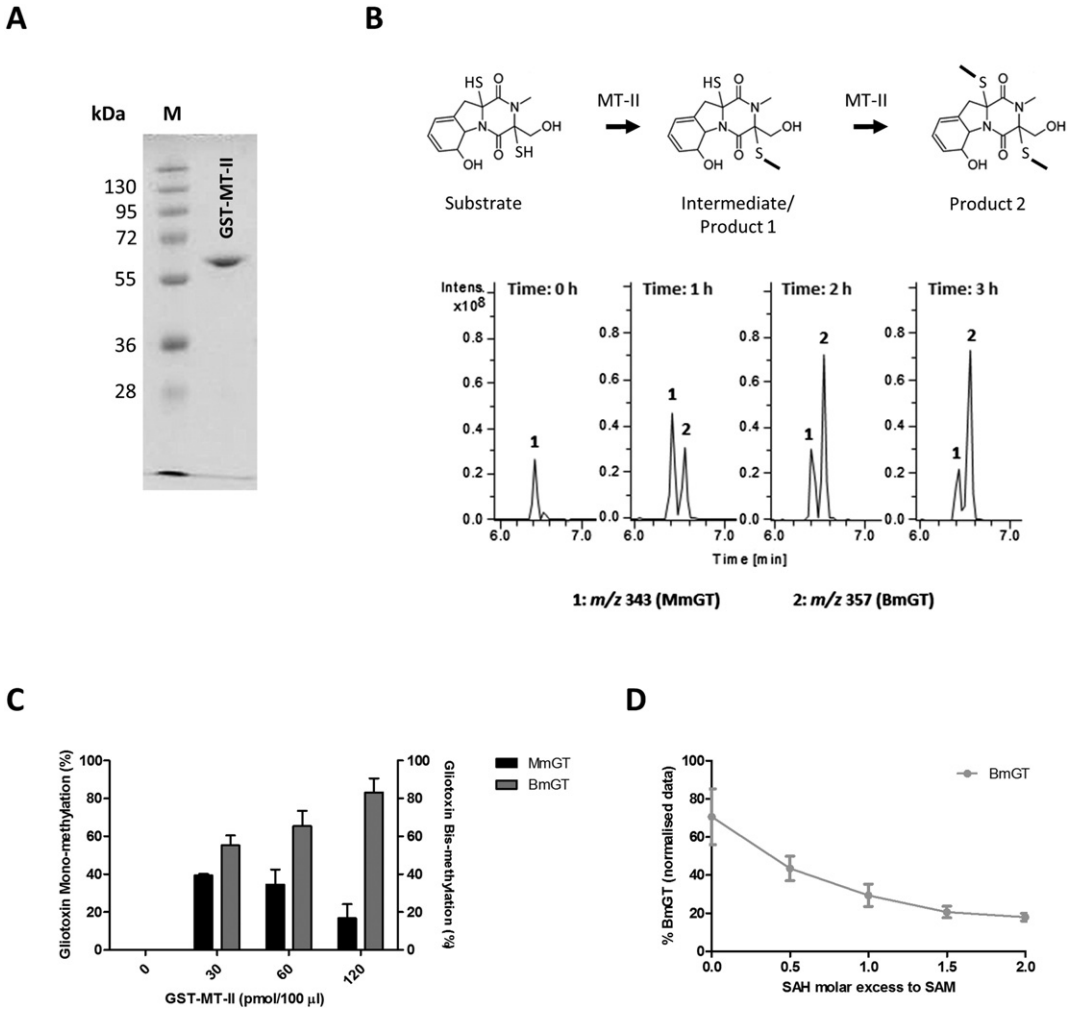

Fig. 5. Expression and activity analysis of recombinant MT-II. (A) SDS-PAGE analysis of recombinant MT-II-GST (59.8 kDa). (B)MT-II-GST mediates the progressive thiomethylation of GT through the formation first of MmGT and then BmGT. (C) The affect of MT-II concentration on the ability to thiomethylate GT. (D) SAH inhibits MT-II catalysed BmGT formation in a concentration dependent fashion.

3.7. Hydrolytic enzyme abundance is increased in response to GT

In *A. niger* exposed to exogenous GT, the abundance of 22 glycoside hydrolases (GHs) was also increased, while that of 3 (An14g04190, An05g02410, An08g05790) was attenuated at 3 h (Table 3). Similarly at 6 h GT exposure, 26 GHs showed increased abundance compared to one that was detectable at lower levels (Table 3). In addition, peptidase abundance showed high levels of dysregulation, whereby 16 showed increased and 4 decreased abundance (at 3 h) and 18 increased with 8 showing reduced abundance at 6 h post-GT exposure (Table 4). The majority of these degradative enzymes were observed at both time-points (57.6% GHs and 43.8% peptidases). From the GHs showing increased abundance after GT exposure at both time points, 83% ($n = 25$) contained signal peptide (SigP), suggesting they could be destined for secretion. Interestingly, the three GHs exhibiting reduced abundance at both time points after GT exposure did not contain SigP. Based on the Carbohydrate-Active Enzymes (CAZy) database (http://www.cazy.org) [30], there are 3 GH families that are characteristic for starch degradation; GH13, GH15 and GH31, which mainly consist of α-amylases, α-glucosidases and glucoamylases. Our data shows the increased abundance of 7 proteins from the GH13 family (AgtC, AamA, AmyC, AgsA, AgtA, GdbA and AgsE), one from the GH15 family (GlaA) and two from the GH31 family (AgdA, AgdB), which could have a role in scavenging starch from the media. Similar to the GHs, most of the peptidases that were increased in abundance after GT exposure (at 3 h and 6 h) contained SigP (68%). Conversely, those peptidases that showed decreased abundance after GT exposure lacked SigP (9 out of 10). This observation further confirms the potential role of these hydrolases in secretory pathways. The proteases with the highest levels of abundance after GT exposure were classified as members of the A1 pepsin family proteases and S53 sedolisin family proteases, based on MEROPS database (merops.sanger.ac.uk) (Table 4). Interestingly, these two families have been observed to be the most abundant under carbon starvation conditions, in *A. niger* [31,32].

4. Discussion

In order to gain an understanding of GT cytotoxicity and reveal new metabolic systems in filamentous fungi, comparative proteomic analysis was carried out on *A. niger* CBS 513.88 in response to sub-lethal levels of GT. Exposure to GT revealed an increased abundance of proteins involved in the methionine cycle and hydrolytic enzymes including GHs and proteases. Closer inspection of the methionine cycle, revealed a dysregulation in the levels of metabolites comprising the cycle and led to the identification and characterization of a methyltransferase (MT-II) responsible for the thiomethylation of exogenous GT (Fig. 2). We further demonstrate that *MT-II* deletion sensitizes *A. niger* to exogenous GT compared to wild-type and thus propose MT-II activity acts as a mechanism of protection against thiol-containing toxic metabolites produced by other fungi.

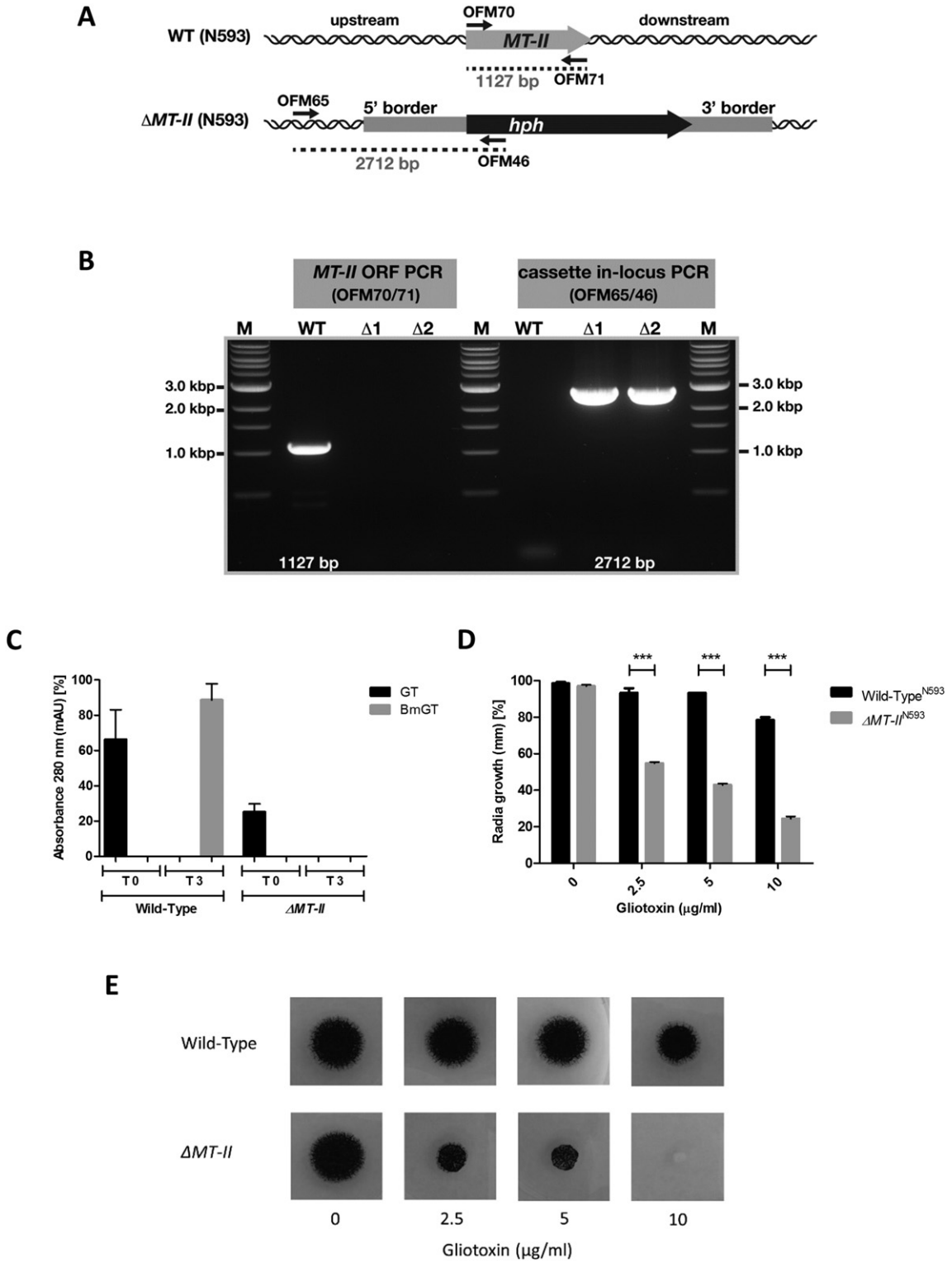

Fig. 6. Increased sensitivity of Δ*MT-II* strain to exogenous GT exposure. (A) A comparative depiction of the *MT-II* WT and *MT-II* deletion loci in *Aspergillus niger* N593 strain. OFM70/71 amplifies *MT-II* open reading frame (ORF), OFM65/46 only amplifies in-locus deletion cassette. Grey shades indicate the border of deletion cassette used for gene replacement/transformation. OFM65 binds 310 bp upstream of hygromycin (*hph*) cassette 5′ border. (B) Gel electrophoresis confirmation of *MT-II* deletion by diagnostic PCR. Δ1 Δ2 represent two independent *MT-II* knock-out strains. Only Δ1 was used for further experiments. *MT-II* ORF (1127 bp) is amplified from WT and missing in Δ1 Δ2. OFM65/46 amplifies in-locus deletion cassette (2712 bp). 50 ng genomic DNA was used as template for PCR reactions. (C) In vivo bis-thiomethylation does not occur in Δ*MT-II* compared to wild-type. (D) Graphical representation of phenotypic analysis shown in Fig. 6E. (E) Phenotypic assay reveals that Δ*MT-II* is highly sensitive to GT compared to wild type.

Dysregulated abundance of proteins involved in cysteine and methionine metabolism, along with sulphur metabolism was observed in *A. niger* upon exogenous GT addition. We hypothesise that the significant decrease in the level of SAM is as a consequence of its role in MT-II-catalysed BmGT formation, and possibly due to the increased abundance of a number of SAM-dependent methyltransferases. In transmethylation reactions, these enzymes catalyse the transfer of methyl groups from SAM to a large variety of acceptor substrates ranging

Table 3
Carbohydrate-Active Enzymes (CAZy) undergoing a significant ($p < 0.05$) change in abundance in *A. niger* CBS 513.88 following exposure to gliotoxin (2.5 μg/ml), relative to methanol solvent control. Data sorted by fold change, in descending order.

Protein description	Fold change (Log_2) 3 h	Fold change (Log_2) 6 h	CAZy Family	Name/Alias	Signal P	Accession
Putative endomannanase; induced by caspofungin	Unique	–	GH76	*dfgC*/*manB*	–	An14g03520
Glucan beta-1,3 exoglucanase	↑ 4.007	↑ 3.686	GH55	*exsG*/*bxgA*	SigP	An01g12450
1,4-alpha-D-glucan glucohydrolase; secreted glucoamylase; most highly expressed at the periphery of colonies; repressed by xylose and induced by maltose; AmyR dependant induction on maltose	↑ 3.667	↑ 3.381	CBM20; GH15	*glaA*	SigP	An03g06550
Beta-mannosidase; glycosyl hydrolase family 2; contains several putative N-glycosylation sites	↑ 3.578	↑ 3.239	GH2	*mndA*	SigP	An11g06540
Alpha-galactosidase; alpha-N-acetylgalactosaminidase variant A; CreA regulated; protein levels influenced by presence of starch; N-glycosylation verified at 6 of 8 potential N-glycosylation sites	↑ 3.335	↑ 3.344	CBM13; GH27	*aglA*	SigP	An06g00170
Glucan 1,3-beta-glucosidase; putative glucanotransferase; induced by caspofungin; highly expressed in germinating conidia	↑ 3.191	↑ 3.352	GH17	*bgtB*/*bgl2*	SigP	An03g05290
Putative 1,3-beta-glucanosyltransferase; predicted signal peptide secretion sequence	↑ 3.045	↑ 2.305	CBM43; GH72	*gelD*	SigP	An09g00670
Putative GPI-anchored glucanosyltransferase; cell wall protein; induced by fenpropimorph; highly expressed upon germination	↑ 3.035	↑ 2.454	GH16	*crhD*/*crh1*	SigP	An01g11010
Putative alpha-galactosidase variant B; expression is induced on xylan; XlnR regulated	↑ 2.994	↑ 3.489	GH27	*aglB*	SigP	An02g11150
Putative alpha-1,6-mannanase	–	↑ 2.807	GH76	–	–	An07g07700
Putative alpha-glucanotransferase	↑ 2.783	↑ 3.534	GH13	*agtC*	SigP	An15g07800
Extracellular alpha-glucosidase pro-protein; the mature form of the enzyme is a heterosubunit protein; AmyR dependant induction on maltose	↑ 2.775	↑ 3.077	GH31	*agdA*/*aglU*; *agdU*	SigP	An04g06920
Putative endoglucanase	↑ 2.773	↑ 2.301	CBM1; GH5	*eglB*	SigP	An16g06800
Putative acid alpha-amylase; abundantly expressed on D-maltose; most highly expressed at the periphery of colonies; repressed by xylose and induced by maltose; AmyR dependant regulation	↑ 2.739	↑ 2.986	GH13	*aamA*/*amyA*	SigP	An11g03340
Alpha-amylase	↑ 2.530	–	GH13	*amyC*	SigP	An04g06930
Ortholog(s) have glucan endo-1,6-beta-glucosidase activity	↑ 2.335	↑ 2.829	GH30	–	SigP	An03g00500
Glycosylphosphatidylinositol-anchored chitinase, similar to ChiA of A. nidulans; expression induced by tunicamycin and DTT; highly expressed in germinated conidia	–	↑ 2.321	GH18	*ctcA*/*cts1*; *chiF*	SigP	An09g06400
Secreted, thermostable beta-galactosidase; XlnR regulated	↑ 2.290	↑ 3.095	GH35	lacA	SigP	An01g12150
Has domain(s) with predicted carbohydrate binding, catalytic activity and role in carbohydrate metabolic process	–	↑ 2.236	GH92	–	–	An14g04240
Putative 1,3-beta-glucanosyltransferase; predicted signal peptide secretion sequence	↑ 2.105	↑ 2.832	GH72	–	SigP	An08g07350
N-acetyl-beta-glucosaminidase	↑ 2.089	↑ 2.538	GH20	*nag1*/*nagA*	SigP	An09g02240
Putative 1,3-alpha-glucan synthase; induced in the presence of cell wall stress-inducing compounds such as Calcofluor White, SDS, and caspofungin	–	↑ 1.942	GH13; GT5	*agsA*	SigP	An04g09890
Mannosyl-oligosaccharide 1,2-alpha-mannosidase	–	↑ 1.925	GH47	*msdS*	SigP	An01g12550
GPI-anchored alpha-glucanosyltransferase; alpha-glucan transferase; induced by caspofungin/alpha-glucanotransferase; alpha-amylase	–	↑ 1.771	GH13	*agtA*	SigP	An09g03100
Putative alpha-glucosidase; AmyR dependant induction on maltose	↑ 1.736	↑ 1.362	GH31	*agdB*	SigP	An01g10930
Putative beta-glucosidase	–	↑ 1.640	GH3	–	–	An15g01890
Putative 1,3-beta-glucanosyltransferase; predicted signal peptide secretion sequence; induced by caspofungin; expressed during germination	↑ 1.370	–	GH72	*gelA*/*gel1*	SigP	An10g00400
Glycogen debranching enzyme; 1,3-alpha-glucan synthase	↑ 1.170	↑ 1.843	GH13	*gdbA*	–	An01g06120
Alpha glucosidase I; expression enhanced by maltose; expression induced by tunicamycin and DTT	↑ 1.166	–	GH63	–	SigP	An15g01420
Putative 1,3-alpha-glucan synthase; moderately induced in the presence of cell wall stress-inducing compounds such as Calcofluor White, SDS, and caspofungin	–	↑ 1.011	GH13; GT5	*agsE*	SigP	An09g03070
1,4-alpha-glucan branching enzyme	↓ 3.458	↓ 2.198	CBM48; GH13	*gbeA*	–	An14g04190
Has domain(s) with predicted cation binding, hydrolase activity, hydrolyzing O-glycosyl compounds activity and role in carbohydrate metabolic process	↓ 1.937	–	GH2	–	–	An05g02410
Ortholog(s) have glycogen phosphorylase activity, role in glycogen catabolic process, response to heat and cell surface, cytoplasm, hyphal cell wall localization	↓ 1.162	–	GT35	–	–	An08g05790

from small metabolites to bio-macromolecules. SAM is a methyl donor and one of the most interconnected metabolites of the cell. It is involved in sensing and stimulating its own biosynthesis [33] through the methionine cycle [34]. The use of SAM as a cofactor for trans-methylation reactions releases SAH, which, by SAH hydrolase, is degraded to Ado and Hcy. Absence of SAH in *A. niger* lysates was evident by LC–MS/MS analysis. This result correlates with the increased abundance of SAH hydrolase in *A. niger* lysates and the hydrolase activity observed after spiking SAH in acid lysates. SAH is known to behave as a potent inhibitor of SAM-dependent MTases (via competitive inhibition of SAM-binding), therefore, in order for an efficient trans-methylation reaction to occur, a decrease in the levels of SAH by SAHase must take place in the cell [35]. We hypothesise that the high SAHase activity observed in the *A. niger* lysates is consequential to elevated abundance and activity of SAM-dependent enzymes, and serves to decrease cellular SAH levels that otherwise would inhibit MTase reactions. A consequence of SAH hydrolysis, is an increase in the levels of Ado and Hcy. High levels of Hcy in the cell have been shown to be deleterious in fungi [36] and humans [37]. In order to decrease this level, fungi can proceed with the next reaction in the methionine cycle, whereby with the aid of methylenetetrahydrofolate as the methyl source [28], Hcy is re-converted to Met or it can be converted to Cys by the reverse transsulfuration pathway involving cystathionine β-synthase (CBS) and cystathionine γ-lyase (CGL) [38]. Indeed, Met levels, produced from Hcy or from the methionine salvage pathway, were elevated after GT exposure compared to methanol control. Met is one of the most important amino acids, as it is necessary for both protein synthesis and cellular trans-methylation reactions. The correlation between the proteomic and metabolite analysis is indicative of methionine cycle dysregulation and increase in Met recycling. It is also likely that Hcy formation facilitates biosynthesis of methionine.

Exogenous GT induces increased abundance of a number of MTases in *A. niger*. Two putative MTases (MT-I and MT-II) were de novo present

Table 4
Peptidases undergoing a significant ($p < 0.05$) change in abundance in *A. niger* CBS 513.88 following exposure to gliotoxin (2.5 μg/ml), relative to methanol solvent control. Data sorted by fold change, in descending order.

Protein description	Fold change (Log_2) 3 h	Fold change (Log_2) 6 h	Peptidase family (MEROPS)	Name/Alias	Signal P	Accession
Pepsin family secreted protease	Unique	–	A1	–	SigP	An18g01320
Sedolisin family secreted protease	↑ 4.034	↑ 4.464	S53.007	–	SigP	An01g01750
Putative serine carboxypeptidase; acid protease	↑ 3.529	↑ 3.496	S10	*pepF*	SigP	An07g08030
Pepsin family secreted protease; only detected under starvation conditions	↑ 3.523	↑ 3.334	A01.080	*pepAb*	–	An01g00370
Carboxypeptidase Y family secreted protease; expression repressed by tunicamycin and DTT	↑ 3.484	↑ 3.152	S10.016	*protF*	SigP	An03g05200
Sedolisin family secreted protease; lysosomal pepstatin-insensitive protease	↑ 3.410	↑ 2.600	S53.007	–	SigP	An03g01010
Sedolisin family secreted protease; lysosomal pepstatin-insensitive protease	↑ 3.325	↑ 3.155	–	–	SigP	An06g00190
Secreted lysosomal Pro-Xaa carboxypeptidase	↑ 3.233	↑ 2.415	S28.004	*protA/EPR*	SigP	An08g04490
Aspergillopepsin A, a pepsin family secreted acid protease; expression increases by growth without ammonia; under carbon control and pH regulation	↑ 2.645	↑ 2.423	A01.016	*pepA*	SigP	An14g04710
Serine-type carboxypeptidase Y family secreted neutral protease	↑ 2.382	↑ 1.657	S10.014	–	SigP	An02g04690
Sedolisin family secreted protease; putative lysosomal pepstatin-insensitive protease	↑ 1.981	↑ 3.330	S53.010	*protB*	SigP	An08g04640
Putative estherase/lipase/thioesterase; acylaminoacyl-peptidase; repressed by growth on starch and lactate	–	↑ 1.970	S09.012	–	–	An09g02830
Ortholog(s) have extracellular region localization	–	↑ 1.868	S10.016	–	SigP	An06g00310
Protein similar to dipeptidyl peptidase II; expression repressed by tunicamycin and DTT	↑ 1.741	–	S28	–	SigP	An12g05960
Ortholog(s) have endoplasmic reticulum localization	↑ 1.625	↑ 1.523	M28.010	–	–	An02g06300
Putative protease	–	↑ 1.378	S09.057	*protC/apsC*	SigP	An04g02850
Vacuolar aminopeptidase Y family secreted protease; expression repressed by tunicamycin and DTT	↑ 1.261	↑ 1.898	M28.001	*ape3*	SigP	An03g01660
Prolyl amino peptidase involved in protein degradation; removes N-terminal proline and hydroxyproline residues from peptides	↑ 1.234	↑ 2.160	S33.008	*papA*	–	An11g04730
Ortholog(s) have dipeptidase activity and cytoplasm localization	–	↑ 1.119	M24.A09	–	–	An05g00050
Protein with similarity to D-stereospecific aminopeptidase; acid protease; expression induced by tunicamycin and DTT	–	↑ 1.079	–	–	–	An16g06750
Putative vacuolar serine proteinase; putative signal sequence for transport into the endoplasmic reticulum; secreted protein	↑ 1.076	–	S8	*pepC*	SigP	An07g03880
Putative zinc aminopeptidase	–	↑ 1.068	M01.007	*apsA*	–	An04g03930
Ortholog(s) have ubiquitin thiolesterase activity, ubiquitin-specific protease activity, role in protein deubiquitination and cytosol, nucleus localization	Absent	–	C12.003	–	–	An02g13920
Ortholog(s) have endopeptidase activator activity and role in proteasomal ubiquitin-independent protein catabolic process, proteasome-mediated ubiquitin-dependent protein catabolic process	Absent	–	T01.984	–	–	An04g01870
Putative metallopeptidase	–	Absent	M24.A11	–	–	An01g14920
Ortholog(s) have endopeptidase activator activity and role in proteasomal ubiquitin-independent protein catabolic process, proteasome-mediated ubiquitin-dependent protein catabolic process	–	Absent	T01.P02	–	–	An04g01800
Ortholog(s) have RNA polymerase II core promoter proximal region sequence-specific DNA binding, cysteine-type peptidase activity, double-stranded DNA binding, mRNA binding, single-stranded DNA binding activity	↓ 2.626	–	C01.084	–	–	An01g01720
Has domain(s) with predicted serine-type carboxypeptidase activity and role in proteolysis	↓ 2.121	–	S10	–	SigP	An05g01870
Putative dipeptidyl-peptidase V; serine-type peptidase	↓ 1.926	–	S09.012	–	–	An16g08150
Putative glutamate carboxypeptidase-like protein; repressed by growth on starch and lactate	↓ 1.154	↓ 1.775	M20.017	–	–	An11g11180
Ortholog(s) have ubiquitin-specific protease activity, role in endocytosis, protein deubiquitination and cell division site, cytosol, nucleus, transport vesicle localization	↓ 1.013	–	C19.A59	–	–	An09g05480
Ortholog(s) have aminopeptidase activity, role in cellular response to drug, chaperone-mediated protein folding, proteolysis and cytosol, extracellular region, fungal-type vacuole lumen, ribosome localization	↓ 1.006	–	M18.A01	–	–	An02g11940

and a PRMT (protein arginine MTase) was increased in abundance after 3 h GT presence. After 6 h GT exposure, MT-I and a tRNA (m5U54)-MTase were uniquely present. The biological significance of this remains unclear, but MTases are responsible for the methylation of DNA, RNA and proteins and also numerous small molecule metabolites and natural products. PRMT6-catalysed methylation is proposed to affect gene regulation by modifying protein-nucleic acid interactions [39] while the methylation of a 5-methyluridine (m5U) residue at position 54 by tRNA (m5U54)-MTases, is a conserved feature in eukaryotic tRNA which, in vitro, influences the fidelity and rate of protein synthesis as well as the stability of tRNA tertiary structure [40]. The altered expression of these MTases possibly highlight the affect GT has on transcription and translation, as noted by O'Keeffe et al. for *A. fumigatus* Δ*gliT* [41].

The two putative MTases have been up-regulated in an *A. niger* library in response to reductive stress agent, DTT [13]. MT-I contains a domain with identity to the ubiquinone-MT (UbiE/COQ5) domain which includes a predicted SAM-binding motif. It was suggested by these authors that DTT affects the electron transport chain as coenzyme Q is part of it. MT-II, on the other hand is the ortholog of an *A. fumigatus* SAM dependent MTase termed GtmA [11]. This MTase has been demonstrated to methylate GT and to play a role in attenuating GT biosynthesis [11]. This homology prompted us to investigate if the same process was occurring in *A. niger*. The in vivo bis-thiomethylation of exogenous GT by *A. niger* was confirmed (Fig. 4A). Thus, GT fed to *A. niger* in liquid cultures must enter the cells in order to be methylated, and the resulting BmGT product is released into the media. *A. nidulans* was also observed to bis-thiomethylate exogenous GT, further emphasizing the ability of GT-naïve fungi to carry out this modification [12]. In vitro thiomethylation of exogenous GT was observed only in *A. niger* lysates obtained from GT-induced cultures, which implies the induction of an enzyme able to catalyse this reaction. Due to the homology of MT-II to GtmA, MT-II was the best candidate protein to carry out this reaction. We successfully expressed the putative methyltransferase MT-II and characterized it biochemically. Using GT and SAM as co-substrates, the enzymatic products of MT-II were identified as MmGT and BmGT. Therefore, MT-II catalyses the sequential methylation at both thiol

groups of GT. Generation of MmGT was observed at shorter reaction times and lower enzyme concentrations indicating that when there is enzyme limitation, only one methyl group is incorporated into GT. It thus appears that mono-methylation by MT-II is a much faster reaction compared to bis-thiomethylation and that this subsequent activity results in a progressive incorporation of methyl groups [11].

In plants, the biological function of thiol methyltransferases (TMTs) has been associated with the detoxification of xenobiotics, for example: reactive thiols produced during the degradation of glucosinolate in cabbage, like thiocyanate and thiosalicylic acid, are detoxified by a TMT that thiomethylates them into non-reactive derivatives [42]. The same role was observed in *Phycomitrella patens*, whereby PpSABATH1 was responsible for the detoxification of thiobenzoic acid [43]. In *A. fumigatus*, GtmA has been demonstrated to play a role in the bis-thiomethylation of endogenously produced GT which in turn attenuates GT biosynthesis [11]. Due to the absence of the GT biosynthetic machinery in *A. niger* and the non-reversible bis-thiomethylation of exogenous GT into an unreactive metabolite, we hypothesise that MT-II is responsible for the detoxification of xenobiotic thiols, and that specifically it represents an ancient defence strategy against ETP-producing fungi occupying identical environmental niches as *A. niger*. Indeed, expression of MT-II only after 3 h GT exposure and not after 6 h, suggests that all exogenous GT has been methylated before 6 h and there is no need for this MTase to be expressed after this time. Furthermore, conclusive and in agreement with the aforementioned hypothesis, the observation that Δ*MT-II* is unable to methylate exogenous GT and is highly sensitive to GT compared to wild-type, positions this enzyme as a requirement for *A. niger* survival in the presence of thiol containing toxic metabolites.

A significant increased abundance of hydrolytic enzymes was also observed after exposure to GT (at 3 h and 6 h) in *A. niger*. The up-regulation of glycoside hydrolases (GHs) and proteases has been demonstrated in transcriptomic and proteomic analysis of *A. niger* and *A. nidulans* grown under carbon starvation [32,44–46]. Like GT exposure, carbon starvation is a type of stress that triggers a response by filamentous fungi. Munster et al. [45] proposed that the increased number of genes encoding CAZymes observed under carbon limitation could have a scouting role during starvation, releasing sugars from complex polysaccharides. Of the glycoside hydrolases up-regulated in response to GT, 22 out of 30 were identified in the extracellular proteome of *A. niger* grown on carbon limitation. These include GlaA, AamA, AgdA, CrhD, BgtB, GelD, BxgA, AglA, NagA, An03g00500, MsdS, EglB, AgdB, AglB, An14g04240 (no SigP), AmyC, MndA, An07g07700 (no SigP), GelA, DfgC (no SigP), An08g07350, and CtcA [46]. Due to the presence of a N-terminal signal peptide (SigP) in the majority (83%) of GHs up-regulated after GT addition, we hypothesise that this response is a consequence of a carbon limitation or a glucose demand induced by GT, whereby *A. niger* responds by synthesizing these enzymes in order to breakdown complex carbohydrates. In agreement with this hypothesis, some of these GHs; AamA, AmyC, GlaA, AgdA and AgdB, have been predicted to have a role in starch degradation and are under the control of AmyR, which is the transcriptional regulator of starch degrading enzymes [47]. Also, AglB and LacA appeared to be regulated by both XlnR and AraR under D-xylose treatment while AglA and AgsA were solely regulated by XlnR and AraR, respectively, under SEB (steam-exploded sugarcane bagasse) [48]. Transcriptional activator XlnR controls the regulation of the xylanolytic/cellulolytic system in *A. niger* [49]. AraR is a transcriptional regulator that can interact with XlnR and plays a role in polysaccharide degradation [50].

Interestingly, some of the proteins with increased abundance in response to GT were possibly involved in autolysis or cell wall remodelling. NagA has a role in the degradation of cell wall component chitin [51] and along with An07g07700, was identified in *A. niger* under carbon starvation [32,45]. CtcA (endo-chitinase) is an ortholog of the GPI-anchored *A. nidulans* ChiA, which is thought to remodel the structure of chitin to a more loose arrangement during growth [52]. In addition to the increase in GHs, a number of peptidases also showed increased abundance after GT exposure at both time points. These proteases (68%) also contained a signal peptide indicating a potential extracellular role. Studies on protease activity in *A. niger* are mainly focused in exploiting its naturally high secretion capacity in biotechnology by manipulating *A. niger* strains that are more efficient in recombinant protein production [19]. Extracellular proteases generally play a role in the breakdown of large polypeptides into smaller molecules for absorption by the cell [53]. Thus, protease activation has been shown to occur under nutrient limitation i.e. carbon or nitrogen [32,54].

In this study, we observed that 13 out of 22 peptidases with increased abundance after GT exposure were also expressed in *A. niger* grown under carbon starvation [31,32]. These proteases include An18g01320, An01g01750, PepF (An07g08030), An01g00370, An03g01010, An06g00190, PepA (An14g04710), An02g04690, An08g04640, An09g02830, An12g05960, An03g01660, PepC (An07g03880). Although these authors relate the increase in proteases to an autolytic process observed in ageing cultures during batch cultivation, we hypothesise that autolysis might be occurring to meet nutrient requirements. Also, because a number of proteases lacked a signal peptide (7 out of 22), we suggest that intracellular proteolytic activity is activated during GT exposure. Overall, this data indicates that the response of *A. niger* to GT is very similar to the response under carbon starvation and suggests that GT causes a nutrient requirement which leads to the production of hydrolytic enzymes. Relevantly, it has been observed that the production of CAZymes and proteases depends on functional regulators BcVEL1 and BcLAE1, in *Botrytis cinerea*, which are the *A. nidulans* orthologs of VeA and the SAM-dependent methyltransferase LaeA, respectively [55]. In *A. nidulans*, VeA and LaeA form a trimeric complex with another member of the velvet protein family, VelB, and this complex is responsible for the regulation of secondary metabolism and development [56]. Apart from functionality in secondary metabolism, LaeA in *A. nidulans* also controls expression of carbohydrate metabolism enzymes such as Hülle cell specific α-mutanase, which is involved in establishment of fully mature fruiting bodies [57]. In *Trichoderma reesei*, LAE1 (*A. nidulans* LaeA ortholog) has been shown to regulate the expression of genes involved in lignocellulose degradation whereby in a *lae1* deletion mutant, a number of cellulases and xylanases were no longer expressed. Additionally, XYR1, the cellulase and hemicellulase transcriptional regulator, was shown to be dependent on LaeA and vice versa [58]. The deletion of *vel*1, which is the *T. reesei* ortholog of *A. nidulans veA*, also diminished the expression of xylanases, cellulases and XYR1 when grown on lactose [59]. The *A. niger* ortholog of XYR1, XlnR, was described by Peij et al. as the transcriptional activator of xylanolytic enzymes [49]. Herein, *A. niger* exposed to GT led to an increase abundance in AglB, LacA and AglA, which are under the control of XlnR. In *A. fumigatus*, deletion of *veA* was shown to cause a reduction of protease activity [60]. We conclude that it is possible that the changes observed in the production of hydrolytic enzymes, after GT addition, are mediated by alterations to the Velvet complex.

5. Conclusions

This study provides new insights into the affect of GT on diverse cellular processes in the GT-naïve fungus *A. niger*. Exogenous GT is converted to BmGT, in vivo, by *A. niger*. Exogenous GT alters *A. niger* methionine cycle (proteins and metabolites) due, at least in part, to depletion of SAM to effect BmGT formation. MT-II is expressed only after GT exposure and is responsible for the exogenous bis-thiomethylation of GT. Recombinant MT-II sequentially methylates rGT to MmGT and then BmGT, and acts to protect *A. niger* against exogenous GT. Hydrolytic enzyme abundance is increased upon exposure to exogenous GT, which may result from transcriptional reprogramming and be indicative of an increased glucose demand. Future work will dissect the interaction between GT exposure and increased GH abundance in *A. niger*.

Glossary

Ado	adenosine
BmGT	bisthiobis(methylthio)gliotoxin
CAZy	carbohydrate active enzymes
CBS	cystathionine β-synthase
CGL	cystathionine γ-lyase
COQ	coenzyme Q
FunCat	functional catalogue
GH	glycoside hydrolase
GliT	gliotoxin (GT) oxidoreductase
GliA	gliotoxin (GT) major facilitator superfamily (MFS) transporter
GST	glutathione *S*-transferase
GT	gliotoxin
Hcy	homocysteine
KEGG	Kyoto encyclopedia of genes and genomes
LFQ	label-free quantitative
MEROPS	the peptidase database
MmGT	monodethiomono(methylthio)gliotoxin
MT or MTase	methyltransferase
OPA	*O*-phthaldialdehyde
PDB	Potato Dextrose Broth
PGC	porous graphitized carbon
PpSABATH1	*Physcomitrella patens* salicylic *a*cid MT, *b*enzoic *a*cid MT and *th*eobromine synthase
PRMT	protein arginine methyltransferase
SAH	*S*-adenosylhomocysteine
SAHase	*S*-adenosylhomocysteinase
SAM	*S*-adenosylmethionine
SEB	steam exploded sugarcane bagasse
SigP	signal peptide
TM	transformation
TMT	thiomethyltransferase
ubiE	ubiquinone
UPR	Unfolded Protein Response

Transparency document

The Transparency document associated with this article can be found, in online version.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jprot.2015.10.024.

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