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The *Neurospora crassa* mutant *NcΔEgt-1* identifies an ergothioneine biosynthetic gene and demonstrates that ergothioneine enhances conidial survival and protects against peroxide toxicity during conidial germination

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ABSTRACT

Ergothioneine (EGT) is a histidine derivative with sulfur on the imidazole ring and a trimethylated amine; it is postulated to have an antioxidant function. Although EGT apparently is only produced by fungi and some prokaryotes, it is acquired by animals and plants from the environment, and is concentrated in animal tissues in cells with an EGT transporter. Monobromobimane derivatives of EGT allowed conclusive identification of EGT by LC/MS and the quantification of EGT in *Colletotrichum graminicola* and *Neurospora crassa* conidia and mycelia. EGT concentrations were significantly ($\alpha = 0.05$) higher in conidia than in mycelia, with approximately 17X and 5X more in *C. graminicola* and *N. crassa*, respectively. The first EGT biosynthetic gene in a fungus was identified by quantifying EGT in *N. crassa* wild type and knockouts in putative homologs of actinomycete EGT biosynthetic genes. *NcΔEgt-1*, a strain with a knockout in gene NCU04343, does not produce EGT, in contrast to the wild type. To determine the effects of EGT *in vivo*, we compared *NcΔEgt-1* to the wild type. *NcΔEgt-1* is not pleiotropically affected in rate of hyphal elongation in Vogel's medium either with or without ammonium nitrate and in the rate of germination of macroconidia on Vogel's medium. The superoxide-producer menadione had indistinguishable effects on conidial germination between the two strains. Cupric sulfate also had indistinguishable effects on conidial germination and on hyphal growth between the two strains. In contrast, germination of *NcΔEgt-1* conidia was significantly more sensitive to *tert*-butyl hydroperoxide than the wild type; germination of 50% (GI_{50}) of the *NcΔEgt-1* conidia was prevented at 2.7 mM *tert*-butyl hydroperoxide whereas the GI_{50} for the wild type was 4.7 mM *tert*-butyl hydroperoxide, or at a 1.7X greater concentration. In the presence of *tert*-butyl hydroperoxide and the fluorescent reactive oxygen species indicator 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate, significantly ($P = 0.0002$) more *NcΔEgt-1* conidia fluoresced than wild type conidia, indicating that EGT quenched peroxides *in vivo*. While five to 21-day-old conidia of both strains germinated 100%, *NcΔEgt-1* conidia had significantly ($P < 0.001$) diminished longevity. Linear regression analysis indicates that germination of the wild type declined to 50% in 35 days, in comparison to 25 days for the *NcΔEgt-1*, which is equivalent to a 29% reduction in conidial life span in the *NcEgt-1* deletion strain. Consequently, the data indicate that endogenous EGT helps protect conidia during the quiescent period between conidiogenesis and germination, and that EGT helps protect conidia during the germination process from the toxicity of peroxide but not from superoxide or Cu^{2+}. Based on an *in silico* analysis, we postulate that *NcEgt-1* was acquired early in the mycota lineage as a fusion of two adjacent prokaryotic genes, that was then lost in the Saccharomycotina, and that *NcEgt-1* catalyzes the first two steps of EGT biosynthesis from histidine to hercynine to hercynylcysteine sulfoxide.

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1. Introduction

Ergothioneine (EGT) is a low-molecular weight thiol in fungi (Fig. 1A) (Hand and Honek, 2005). Tanret (1909) first isolated EGT from *Claviceps purpurea* ergot sclerotia and Barger and Ewins (1911) determined that EGT is the betaine of 2-thiolhistidine. Although EGT is not synthesized by non-fungal eukaryotes, animals and plants acquire EGT from the environment (Ey et al., 2007). Experiments in which EGT was added exogenously to cells that contain EGT suggest that EGT may protect cells from oxidative stress. For example, the sperm in boar semen remained motile in the presence of exogenous H_2O_2 and Cu^{2+} if EGT was also added (Mann and Leone, 1953). Addition of EGT to *C. purpurea* conidia

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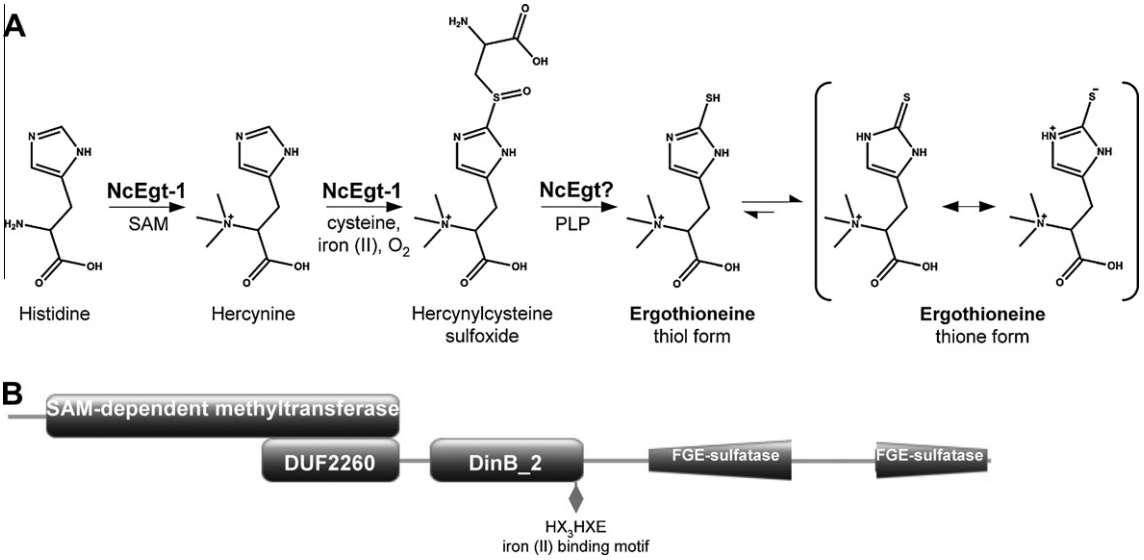

Fig. 1. (A) The structure of ergothioneine (EGT) and the proposed EGT biosynthetic pathway in *Neurospora crassa* and other fungi, and (B) an *in silico* annotation of *NcEgt-1*, which is required for EGT biosynthesis in *N. crassa*.

enhanced germination and germ tube elongation, and protected conidia from the adverse effects of H_2O_2 (Garay, 1956a,b).

EGT differs from the cysteine-based thiols including the tripeptide thiol glutathione (GSH) in that EGT is predominantly a thione tautomer rather than a thiol (Fig. 1A) at physiological pH (Hand et al., 2005). In addition, at pH 7, EGT has a higher redox potential ($E'_0 = -60$ mV) than GSH ($E'_0 = -250$ mV), which makes EGT less susceptible to auto-oxidation and consequently more stable in an aerated aqueous solution (Hand and Honek, 2005; Hartman, 1990).

Observational studies indicate that EGT is synthesized only by fungi (except in the Saccharomycotina), and by bacteria in both the Actinomycetales (Dubost et al., 2007; Genghof, 1970; Melville et al., 1956; Seebeck, 2010) and in at least some members of the cyanobacteria (Pfeiffer et al., 2011). The concentration of EGT in filamentous fungi appears to be variable, depending on species, tissue, and perhaps method of detection. Conidia-free honey-dew produced by *C. purpurea* during infection in rye contains 2–5 μg EGT per μl, (Garay, 1956b), a concentration equivalent to 8.7–22 mM EGT. EGT was quantified at 2–5 μg mg^{-1} of the dry weight of *C. purpurea* sclerotia. However, Heath and Wildy (1956) established that EGT in *C. purpurea* is primarily in conidia rather than in mycelia. In *Neurospora crassa*, EGT concentrations have been estimated from 466 to 850 ng mg^{-1} dry weight (Genghof, 1970; Melville et al., 1956). Dubost et al. (2007) reported a range of EGT concentrations in edible mushrooms from 210 ng mg^{-1} dry weight to the highest mean concentration of 2600 ng mg^{-1} dry weight in the oyster mushroom, *Pleurotus ostreatus*.

Plants acquire EGT through their roots, or perhaps less frequently from fungal and actinomycete symbionts (Melville and Eich, 1956; Park et al., 2010). Animals acquire EGT from food. Some plant foods including garlic, beans, wheat and oats are comparatively EGT-rich, with 3–4.5 ng mg^{-1} fresh weight (Ey et al., 2007). The concentration of EGT in animal tissue is partly diet-dependent (Melville et al., 1955). However, animals have an EGT transporter, OCTN1 in humans (Gründemann et al., 2005), and EGT is concentrated from approximately 2–133 ng mg^{-1} fresh weight in specific tissues, especially in seminal fluid, erythrocytes, kidney, liver and eye cornea tissue (Melville et al., 1954; Shires et al., 1997). Although the role of EGT in animal tissue is unknown, the literature suggests that it may be involved as an antioxidant (Akanmu et al., 1991; Hartman, 1990) in seminal fluid (Mann et al., 1963), red blood cells (Melville et al., 1955), and in the following tissues: skin (Markova et al., 2009); kidney (Deiana et al., 2004), liver (Deiana et al., 2004), brain (Song et al., 2010), and eyes (Shires et al., 1997).

Because EGT has been classified as a thiol, there have been numerous *in vitro* studies on its activity, particularly in comparison to GSH. EGT, at the physiological concentrations of one to two mM in certain animal tissues scavenges the hydroxyl radical ($HO^{\bullet}$) more efficiently than GSH (Akanmu et al., 1991); consequently, EGT prevents the $HO^{\bullet}$-mediated oxidation of α-keto-γ-methiolbutyric acid (Franzoni et al., 2006). In addition, EGT can serve as an effective scavenger of hypoclorous acid (HOCl), thereby preventing the oxidation of α_1-antiproteinase (Akanmu et al., 1991). EGT also has peroxynitrite ($ONOO^-$ and ·ONOOH) scavenging capacity, and inhibited oxidation by $ONOO^-$ of both purified calf thymus DNA and of DNA in a neuronal hybridoma cell line (Aruoma et al., 1999). EGT provided better protection than GSH against oxidation of α-keto-γ-methiolbutyric acid by ONOOH and peroxyl radical (ROO·) (Franzoni et al., 2006). EGT also significantly blocked the inhibitory effects of singlet oxygen ($^1O_2$) generation by the photosensitive dyes Rose Bengal and phenosafranin (Hartman et al., 1990). *In vitro*, EGT forms complexes of 2 mol of EGT to 1 mol of either Cu^{2+} or other metals (Motohashi et al., 1976). As a consequence, EGT also inhibited the Cu^{2+}-mediated oxidation of oxyhemaglobin, and of DNA and bovine serum albumin by the formation of stable EGT–copper ion complexes (Akanmu et al., 1991; Zhu et al., 2011).

The biosynthesis of EGT in fungi has been studied in *N. crassa* (Askari and Melville, 1962; Ishikawa et al., 1974; Melville et al., 1959; Reinhold et al., 1970) and in *C. purpurea* (Heath and Wildy, 1956, 1958; Wildy and Heath, 1957). EGT biosynthesis starts with the triple methylation of histidine via a *S*-adenosyl methionine (SAM)-dependent methyltransferase to hercynine (histidine betaine). The next step in the pathway requires O_2 and Fe^{2+} for the sulfoxidation of hercynine to hercynylcysteine sulfoxide (Ishikawa et al., 1974). Finally, a pyridoxal phosphate-requiring enzyme converts hercynylcysteine to ergothioneine.

Studies with the actimomycetes *Mycobacterium tuberculosis* and *M. smegmatis* (Genghof and Van Damme, 1968, 1964) suggest that the EGT biosynthesis pathway in actinomycetes is similar to that in fungi. Recently, a five-gene cluster for EGT biosynthesis in

Mycobacterium avium was identified and designated as *egtA* through *egtE* (Seebeck, 2010). The biochemical function of the *Mycobacterium tuberculosis* homolog of *egtA* (Harth et al., 2005), the *M. smegmatis* homologs of *egtB*, *egtC*, and *egtD*, and the *Erwinia tasmaniensis* homolog of *egtE* (Seebeck, 2010) were assessed using heterologous protein expression with potential substrates. The gene cluster functional prediction is as follows: *egtA*, γ-glutamyl cysteine synthase; *egtB*, formylglycine-generating enzyme (FGE)-sulfatase; *egtC*, class-II glutamine amidotransferase; *egtD*, SAM-dependent methyltransferase; and *egtE*, pyridoxal 5-phosphate (PLP)-dependent β-lyase.

In this study, the monobromobimane derivative of EGT was used to conclusively identify EGT by LC/MS, and to quantify the higher concentrations of EGT in *Colletotrichum graminicola* and *N. crassa* conidia than in mycelia. We identified the first fungal biosynthetic EGT gene, *NcEgt-1*, by quantifying EGT in *N. crassa* wild type and knockouts in putative homologs of actinomycete EGT biosynthetic genes. The ergothioneine-minus mutant was used to determine the role of EGT in a filamentous fungus. Finally, we used full-genome sequences to investigate the evolution of fungal NcEgt-1 homologs.

2. Material and methods

2.1. Fungal strains and culture conditions

All strains were maintained in silica gel at −70 °C (Nakasone et al., 2004).

2.1.1. Colletotrichum graminicola wild type M1.001

To produce falcate conidia, cultures were seeded with silica stocks onto Petri dishes with oatmeal agar (30 g oatmeal flour and 20 g agar/l) and incubated in constant light at 24–26 °C for 7 days. To collect conidia for biochemical analyses, sterile distilled water was added to the dish and a bent metal rod was used to dislodge conidia from the agar surface. After the conidial suspension was centrifuged at 16,000*g* for 1 min, the supernatant was removed, and the cell pellet was flash-frozen in liquid nitrogen and lyophilized.

For production of mycelia, dishes with potato dextrose agar (PDA) (Difco Laboratories, Detroit, MI, USA) were seeded with stock cultures. Agar plugs were taken from the margin of 7-day-old colonies and inoculated into 125 ml-flasks containing 30 ml of Fries medium. (30 g sucrose, 5 g ammonium tartrate, 1 g ammonium nitrate, 1 g potassium dihydrogen phosphate, 1 g sodium chloride, 250 mg anhydrous magnesium sulfate, 130 mg calcium chloride dihydrate, and 1 g yeast extract/l). Stationary flasks were incubated for 5 days at 30 °C in the dark. Mycelial mats were collected onto cheesecloth under vacuum, rinsed with distilled water, flash-frozen in liquid nitrogen and lyophilized.

2.1.2. Neurospora crassa

N. crassa wild type OR74A (FGSC 9013, *mat A*) and knockout mutants (*mat A*) of the homologs of *M. avium egtB* (FGSC strain 19115, gene NCU04343, 25% amino acid identity to *M. avium* gene), *egtC* (FGSC 19390, gene NCU01256, 34% identity) and *egtD* (FGSC 17907, gene NCU07917, 29% identity) were obtained from the Fungal Genetics Stock Center (Colot et al., 2006; McCluskey, 2003).

For production of conidia, slants with Vogel's agar (1X Vogel's salts, 15 g sucrose, 2 g ammonium nitrate and 15 g agar/l) were inoculated with stock cultures, and incubated under fluorescent light at 24–26 °C. Conidia were harvested from 5 to 10-day-old cultures by adding sterile distilled water to the slant, vortexing the culture tube, and filtering the conidia through Miracloth to remove any mycelia. The conidial suspension was transferred to a 1.5 ml microfuge tube, and pelleted at 16,000*g* for 1 min. When required, the pellet was either resuspended in water or 0.01% Tween 20 and the conidial concentration was adjusted using a hemocytometer. For the data in Table 1, conidia were flash-frozen in liquid nitrogen, lyophilized, and stored at −70 °C.

To produce mycelia for the data shown in Table 1, Petri dishes with Vogel's agar were inoculated with conidia from slants (5–10-day-old) and incubated overnight at 24 °C in the dark. Thirty ml Vogel's broth in 125 ml-flasks were inoculated with agar plugs (6 mm diam); flasks were incubated stationary for 24 h at 30 °C in the dark. Mycelia were then harvested as indicated in section 2.1.1.; the quantity of biomass produced by *N. crassa* after 24 h is similar to that produced by *C. graminicola* after 5 days.

2.1.3. Saccharomyces cerevisiae

The yeast strain BY4742 was purchased from Open Biosystems (Thermo Fisher Scientific, Waltham, MA, USA). The yeast culture was grown at 30 °C in YPD broth in a rotary shaker at 200 rpm. After overnight incubation, 1 ml of the cell suspension was pelleted by centrifugation for 2 min at 16,000*g*, washed twice with sterile distilled water, and immediately frozen and lyophilized.

2.2. Identification of HPLC-purified EGT by liquid chromatography-mass spectrometry (LC/MS)

2.2.1. Solubilization of low molecular weight thiols

Except when indicated otherwise, 2 ml cryogenic vials containing approximately 10 mg of either lyophilized conidia, mycelia, or yeast cells, 550 µl of 10% perchloric acid (PCA; Sigma–Aldrich, St. Louis, MO, USA) and 250 µl of 0.5-mm-diam glass beads (BioSpec, Bartlesville, OK, USA), were topped-off with argon gas to prevent oxidation during tissue disruption. Vials were shaken with a FastPrep system (BioSpec) for eight cycles of 30 s each, with 2 min cooling on ice between each cycle. After an aliquot of 100 µl of crude cell extract was transferred to a new tube, 20 µl of dichloromethane (DCM; Sigma–Aldrich) was added to remove lipids from the 10% PCA. After the DCM-extract mixture was vortexed and

Table 1
Concentration of the low-molecular weight thiols glutathione (GSH) and ergothioneine (EGT) in conidia and mycelia of *Colletotrichum graminicola* and *Neurospora crassa*.

Species	Strain	Stage[a]	GSH (nmol mg^{-1} protein) ± SE[b]	EGT (nmol mg^{-1} protein) ± SE[b]
C. graminicola	Wild type	Conidia	1.3 ± 0.2	11.6 ± 0.9
C. graminicola	Wild type	Mycelia	3.8 ± 0.6	0.7 ± 0.04
N. crassa	Wild type	Conidia	3.0 ± 0.1	25.4 ± 1.3
N. crassa	Wild type	Mycelia	5.5 ± 0.1	5.2 ± 0.4
N. crassa	NcΔEgt-1	Conidia	4.2 ± 0.1	0.0 ± 0.0
N. crassa	NcΔEgt-1	Mycelia	6.0 ± 0.3	0.0 ± 0.0

[a] Conidia from *C. graminicola* and *N. crassa* were harvested from 7-day-old cultures on oatmeal and Vogel's agar plates, respectively. Mycelia from *C. graminicola* were harvested from 5-day-old cultures in Fries broth and mycelia from *N. crassa* were harvested from 1-day-old cultures in Vogel's broth. After lyophilized tissue was disrupted in 10% perchloric acid (PCA), the PCA-soluble fraction was cleared of lipids, derivatized with monobromobimane, separated by reverse phase HPLC and quantified using a fluorometric detector.

[b] Data are the means ± SE of three replicates.

centrifuged at 14,000*g* for 20 min at 4 °C, the supernatant was transferred to a new tube. The samples were either used immediately or stored at −70 °C.

2.2.2. Separation of low molecular weight thiols by reverse-phase HPLC with electrochemical (EC) detection

Standard stocks of reduced glutathione (GSH; 1 mg ml^{-1}), oxidized glutathione (GSSG; 2 mg ml^{-1}), cysteinyl-glycine (0.579 mg ml^{-1}), cysteine (0.571 mg ml^{-1}) and ergothioneine (0.746 mg ml^{-1}) were prepared in 10% PCA and stored at −70 °C. Reagents were obtained from Sigma–Aldrich. Stocks were diluted 1:80, except for ergothioneine which was diluted 1:5, and then serially diluted 1:2 in 10% PCA. Standards were either used immediately or stored at −70 °C in aliquots for single use. Samples prepared as described in Section 2.2.1 were diluted 1:5 in 10% PCA. The system had a Waters (Milford, MA, USA) 510 dual pump, a 717 plus autosampler, and a Synergi 4 μm Hydro-RP 80A column (4.6 × 250 mm with a 2 × 3 mm precolumn; Phenomenex, Torrance, CA, USA). Samples and standards were eluted isocratically for 45 min run at a flow rate of 0.9 ml/min with a mobile phase of 25 mM sodium phosphate, 100 μM sodium octanesulfonate, and 1.8% acetonitrile (ACN) with the pH 2.7 adjusted with phosphoric acid. Typically 10 μl of samples and of standards were injected. The injected volume contained thiols from a sample that originally had 7–10 μg of solubilizable protein. Oxidizable compounds were detected using an ESA Coulochem II electrochemical detector equipped with a model 5040 analytical electrochemical cell and a model 5020 guard cell (ESA, Chelmsford, MA, USA). The detector potentials were set at 1400 mV, and the guard cell at 975 mV with a full-scale output of 1 μA. The Empower 2 Chromatography Data Software (Waters) was used to determine the area of samples and standards. All linear regressions of standards had $R^2 > 0.999$. Low molecular weight thiols were identified by co-elution of peaks in samples that were spiked with an approximately equal quantity of a standard, and by retention times of peaks in samples compared to standards.

2.2.3. Preparation of monobromobimane (mBBr) derivatives of GSH and EGT, their separation on HPLC with fluorometric detection, and identification of mBBr-EGT by LC/MS

The GSH and EGT standards and the cell-free lysates in 10% PCA were derivatized using THYOLITE monobromobimane (mBBr; Calbiochem, La Jolla, CA, USA); unbound mBBr is weakly fluorescent but mBBr-thiol esters are strongly fluorescent. Derivatization was performed in reduced light in dark tubes to prevent photobleaching. Immediately after 100 μl aliquots of standards and samples were adjusted to pH 7.0–7.5 with 80 μl of 2 M KOH in saturated $K_2B_4O_7$, 10 μg mBBr in 10 μl ACN were added. The mixtures were incubated in the dark for 1 h at 24 °C. Afterward, 2.5 μl of 85% phosphoric acid was added to the mixtures to adjust the pH to 3.0–3.5. Samples were centrifuged at 16,000*g* for 4 min and the supernatant was transferred to a new tube.

For LC/MS analysis, aliquots of mBBr-EGT and mBBr-conidial lysate were injected onto the same HPLC column and apparatus indicated in the section 2.2.2 and eluted with a flow rate of 0.9 ml/min for 75 min with a linear gradient of buffer A (50 mM ammonium acetate in water) and buffer B (50 mM ammonium acetate in 75% ACN) with the following program: 0–40 min, 10% B; 40–45 min, 16.7% B; 45–55 min, 100% B; and 60–75 min, 10% B. The mBBr-derivatives were visualized with a 2475 multi λ fluorescence detector (Waters) with excitation/emission wavelengths of 394/490 nm and gain = 1.

The mBBr-EGT peak of the standard (~40 nmol) and the putative mBBr-EGT peak from conidial (~32 nmol) samples were collected, dried by vacuum centrifugation, resuspended in 50 μl 10% ACN, and stored at −70 °C. Before LC/MS analysis, 10 μl per sample was run quantitatively as indicated above to confirm the purity of EGT-collected peaks. To verify the molecular mass of EGT-mBBr derivatives, 5 μl with ~3 nmol were analyzed using a Thermo Surveyor HPLC system with a 3.5 μm XBridge C18 column (2.1 × 150 mm; Waters), a Micro AS autosampler and a LTQ/Orbitrap mass spectrometer (Thermo Fisher Scientific). HPLC was performed at a constant flow rate of 50 μl/min using a 30 min linear gradient. Mobile phase A was 0.1% formic acid in water, and mobile phase B was 0.1% formic acid in ACN. The gradient was as follows: 0–5 min, 1% B; 5–20 min, 20% B; 20–25 min, 50% B; 25–28 min, 1% B; and a final 1% B for 2 min.

The MS full scans were performed over an *m/z* of 250–1800 at a 30,000 resolution. The ionization voltage was 5.00 kV, capillary temperature was 250 °C and capillary voltage was 45 V. Collision-induced decomposition (CID) was used to fragment the $[M + H]^+$ ions with a normalized collision energy of 35%. MS data were processed and visualized using the Xcalibur 2.0 software (Thermo Fisher Scientific). The mBBr-EGT derivative has an expected molecular mass of 419.49 (271.10 mBBr + 229.29 EGT = 500.399–1.00 H – 79.90 Br = 419.49). Because the mass spectrometer was operated in the positive ion electrospray mode, the expected molecular mass of a single charged ion is 420.49.

2.3. Quantification of mBBr-GSH and mBBr-EGT by HPLC with fluorometric detection

Fresh standards were prepared for each trial. The serial dilutions indicated in Section 2.2.2 were reacted with mBBr as described in Section 2.2.3. The mBBr-derivatives of sample lysates were quantified by injecting either 10 μl containing thiols from conidia and mycelial lysates that had 20–30 μg of solubilizable protein into the HPLC system. Chromatography was carried out as indicated in Section 2.2.3 with the elution gradient and fluorometric detection system. Peak identification and quantification was performed as indicated in Section 2.2.2 for the HPLC-EC system.

2.4. Quantification of sample protein

Except when indicated otherwise, EGT and GSH were normalized to soluble protein in the samples, and expressed as nmol mg^{-1} protein in the cell extract. Protein was assayed using the BCA (Thermo Scientific Fisher) method with modifications. Briefly, 50 μl of the lysed cells in 10% PCA were transferred to a new tube using a wide bore pipette tip (2 mm diameter) and centrifuged at 16,000*g* for 10 min to precipitate all protein. After 25 μl of the supernatant was discarded, the pH of the remaining cell suspension was raised between pH 7.5 and 8.0 with 25 μl of 1.5 M NaOH. After 75 μl of a buffer solution of 3.3% SDS and 0.42 M Tris was added for a final concentration of 0.3 M NaOH, 2% SDS and 250 mM Tris pH 7.5, the mixture was vortexed, incubated at 60 °C for 2 h, and resuspended as needed to maintain the crude cell lysate in suspension. Incubated lysates were recentrifuged at 16,000*g* at room temperature for 3 min, and two-50 μl aliquots were assayed for protein concentration by incubating samples at 30 °C for 30 min. BSA standards were prepared as samples with 0.3 M PCA, 0.3 M NaOH, 2% SDS, and 250 mM Tris–HCl, pH 7.5.

2.5. Phenotypic characterization of NcΔEgt-1 in comparison to the wild type

2.5.1. Percentage germination of N. crassa macroconidia

Except when indicated otherwise, germination assays were conducted with 1.4×10^6 conidia in 50 μl droplets in 1 cm diameter multi-well slides (Carlson Scientific, Peotone, IL, USA). Amendments were freshly prepared and filter-sterilized. Except when

indicated otherwise, all solutions contained the indicated amendment in Vogel's broth in 0.01% Tween 20. Multiwell slides were incubated at 32 °C in moist chambers in the dark for the indicated time period. After incubation, conidia were fixed in 100 μl of lactophenol aniline blue (w/v: 20% phenol crystals, 20% lactic acid, 40% glycerol, and 0.05% aniline blue) and percentage germination of 100 conidia was assessed for three replicates per treatment.

Because *N. crassa* conidia aggregated when incubated in Vogel's broth amended with cupric sulfate, germination assays were conducted on agar media. A buffer with a final concentration of 5 mM 2-(N-morpholino) ethanesulfonic acid (MES pH 6.1; Sigma) was added to all experiments with copper amendments.

For the effect of conidial age on germinability, Vogel's slants were inoculated biweekly with 7-day-old conidia for three months so that conidia from 2 to 11 weeks old were tested in the same experiment. Data were collected in three independent trials and were analyzed as a general linear model with trial date as a block effect.

2.5.2. Quantification of percentage of conidia forming reactive oxygen species

5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA) (Molecular Probes, Invitrogen, Carlsbad, CA, USA) was stored at −20 °C in DMSO. Conidia as described in section 2.1.2. were incubated in Vogel's broth in 0.01% Tween 20, and either 0 or 7 mM *tert*-butyl hydroperoxide. Multiwell slides were incubated at 32 °C in moist chambers in the dark for 40 min. After 5 μl carboxy-H2DCFDA was gently mixed into the droplet for a final concentration of 10 μM, conidia were incubated at 23 °C in the dark for an additional 40 min. Then conidia were examined on a Leica DM 5000B epifluorescent microscope with a GFP cube (excitation 470/40, emission 525/50). The number of fluorescing conidia and the total number of conidia under differential interference contrast (DIC) were counted immediately in random fields of view using the 40X objective. Fluorescence of a minimum of 140 conidia per replicate in each of three separate trials was assessed. For microscopic images, conidia were photographed with the 20X objective with gain = 3 and gamma = 0.68. All DIC and epifluorescent images were taken for 110 and 500 ms, respectively. The epifluorescent images were over-exposed in order to show the most differences between all treatments.

2.5.3. Quantification of hyphal growth

For the experiment on hyphal elongation in Vogel's agar either with or without ammonium nitrate, Petri dishes (100 mm diam) were inoculated on the edge of the dish with a 4 mm diam plug of 1-day-old mycelia; plugs were taken from cultures with the same media. After incubation of the cultures at 32 °C in the dark for 12 h, hyphal elongation was measured every 2 h for 12 h.

For the quantification of hyphal biomass in copper-amended media, Petri dishes (100 mm diam) with Vogel's broth amended with 0.01% Tween 20, 5 mM MES buffer (pH 6.1), and either 0 or 4.25 mM cupric sulfate were inoculated with a 4 mm diam plug of 1-day-old mycelia; plugs were taken from cultures with the same media. After incubation of the cultures at 32 °C in darkness for 20 h, mycelia was collected with vacuum onto 25 mm diameter nylon membranes with 20 μm pores (Micron Separations Inc., Westboro, MA), rinsed with water, lyophilized, and weighed for determination of net growth.

2.6. Statistical analysis

All experiments were repeated to establish reproducibility of results and contained at least three independent, biological replicates. Data were checked for heteroscedasticity and were transformed before analysis if the assumption of homogeneity of variance ($P < 0.05$) was violated. Data were analyzed using the JMP8 software (SAS Institute, Cary, NC, USA). Means ± the standard error of the mean (SE) are shown in the text. SE are included in all figures, but are often too small to be seen.

Categorical treatment variables were analyzed by ANOVA. To analyze hyphal growth, data from each replicate were analyzed as a linear regression ($R^2 > 0.99$) and then slopes were compared with a two-way ANOVA.

Response data that could be linearized by transformation were transformed and then analyzed by a general linear model. For analysis of the menadione experiment, the fraction germination was transformed as $\sqrt{(1\text{-fraction germinated}^2)}$ ($R^2 = 0.93$). For the effect of *tert*-butyl hydroperoxide, for each strain, the fraction germinated at those concentrations in which all replicates germinated between 1% and 99% inclusive were logit-transformed; consequently, for each replicate, there was a linear regression of the logit germinated at four concentrations of *tert*-butyl hydroperoxide (2–3.5 mM for *NcΔEgt-1* and 3–4.5 mM for wild type) with $R^2 = 0.96$ and 0.87, respectively ($n = 12$). The *P*-values of the difference between the intercepts (strain effect) and the slopes (strain * *tert*-butyl hydroperoxide concentration interaction effect) are indicated. To linearize the data on the effect of conidial age on germinability, data were transformed as log (% germinated + 1) ($R^2 = 0.93$ and 0.89 for wild type and *NcΔEgt-1*, respectively).

3. Results

3.1. Ergothioneine is identified as the major low molecular weight thiol in C. graminicola conidia and is confirmed as a major low molecular weight thiol in N. crassa conidia

C. graminicola cell-free lysates of conidia and mycelia were examined for reduced glutathione (GSH), oxidized glutathione (GSSG), cysteinyl-glycine, and cysteine using HPLC with electrochemical detection. GSH was detected in all tissues at 3.2 ± 0.6 nmol mg^{-1} protein (equivalent to 206 ± 37 ng GSH mg^{-1} dry weight) and 3.4 ± 0.6 nmol mg^{-1} protein (equivalent to 215 ± 40 ng GSH mg^{-1} dry weight) in 7-day-old fresh conidia, and mycelia grown in Fries medium, respectively. Neither GSSG, cysteinyl-glycine nor cysteine were detected. We did not assay for γ-glutamyl-cysteine.

The *C. graminicola* conidial samples had a distinct, well-separated, oxidizable peak that eluted at 25.5 min (Fig. 2B). An EGT standard eluted at the same retention time as the conidia-enriched peak (Fig. 2A), and coeluted when conidial extracts were mixed with the EGT standard (Fig. 2C). Based on the EC-detector, the EGT in mycelia was quantifiable but the concentration was reduced compared to the conidial lysates (Fig. 2D). Compared to the GSH standards, the same quantity of EGT only produced approximately 2% of the redox signal, i.e., the relative response factor for the EC-detector for GSH/EGT was 50.

To provide additional evidence that the conidial-enriched peak was a thiol and was EGT, conidial and mycelial lysates from *C. graminicola* and from *N. crassa*, as well as EGT and GSH standards, were derivatized with the fluorochrome monobromobimane (mBBr). mBBr-conjugates emit at 490 nm whereas unconjugated mBBr is comparatively non-fluorescent (Fahey and Newton, 1987). After separation by reverse phase HPLC, the derivatives were eluted with a linear gradient and quantified by fluorescence. GSH and EGT standards derivatized with mBBr produced a well resolved chromatogram each with a single sharp peak (Fig. 3A); negative controls indicated the peaks marked as mBBr were unconjugated mBBr (Fig. 3). Chromatograms from *C. graminicola* and *N. crassa* wild type conidia and mycelia were similar; results from *N. crassa* are shown in Fig. 3B–F. The only low molecular

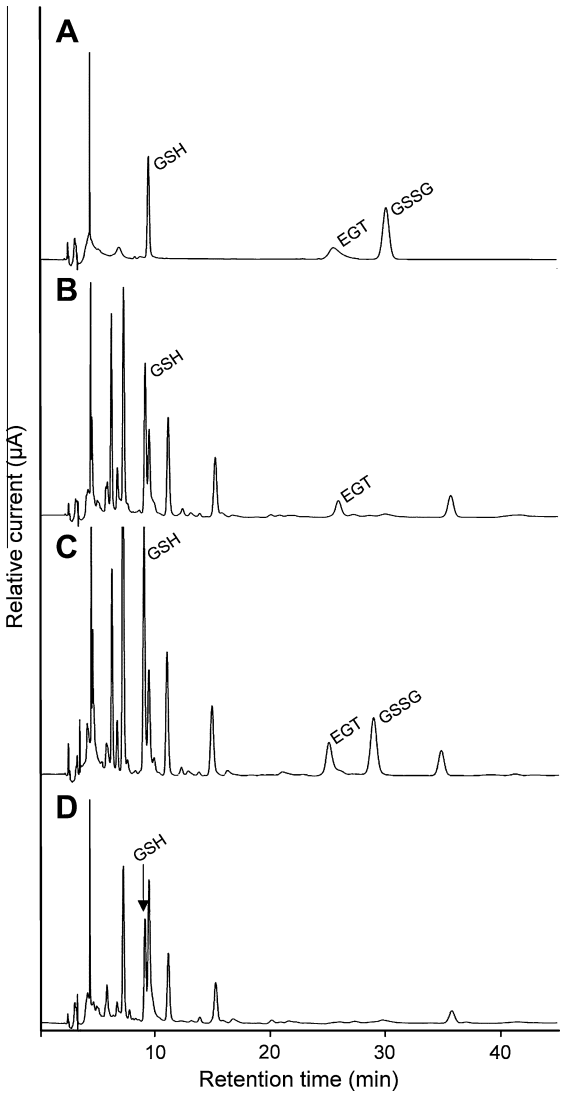

Fig. 2. HPLC separation of 10% perchloric acid-soluble extracts from *Colletotrichum graminicola* and identification of reduced glutathione (GSH), ergothioneine (EGT) and oxidized glutathione (GSSG) with an electrochemical detector at 1400 mV potential and 1μA FS. Chromatogram of a (A) standard with 12.5 pmol GSH at 9.4 min, 20.3 pmol EGT at 25.5 min, and 12.5 pmol GSSG at 30.1 min, (B) conidial extract, (C) the sample in B co-injected with 12.5 pmol GSH, 20.3 pmol EGT, and 12.5 pmol GSSG standards, and (D) mycelial extract. The injected volume contains thiols from conidial and mycelial lysates that contained 7–10 μg of solubilizable protein. In comparison to conidia, mycelia have little EGT.

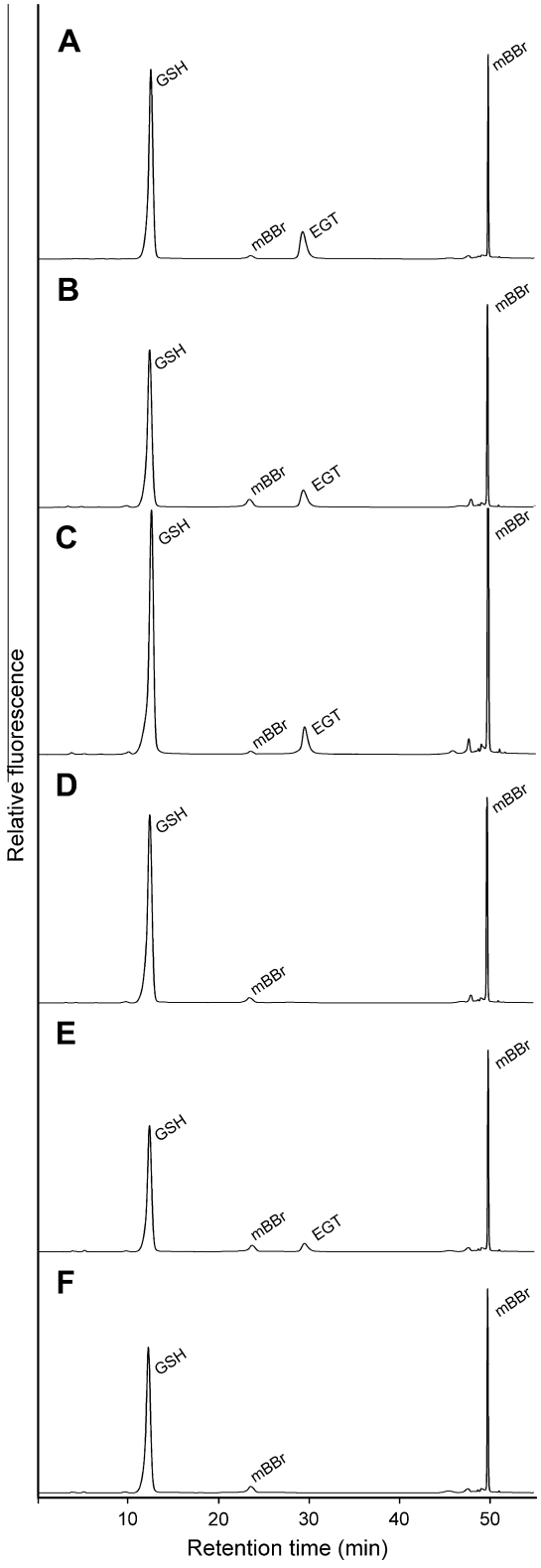

Fig. 3. HPLC separation and identification of low molecular weight thiols from *Neurospora crassa* using fluorometric detection of monobromobimane (mBBr) derivatives at excitation/emission wavelengths of 394/490 nm. Chromatogram of (A) reduced glutathione-mBBr (105 pmol GSH at 12.5 min) and ergothioneine-mBBr (1686 pmol EGT at 29.3 min) standards, and extracts of (B) wild type conidia, (C) half the quantity of the wild type conidial extract in B co-injected with 53 pmol GSH and 843 pmol EGT standards, (D) *NcΔEgt-1* conidia, (E) wild type mycelia and (F) *NcΔEgt-1* mycelia. The injected volume contains thiols from conidial and mycelial lysates that contained 20–30 μg of solubilizable protein. Negative controls (data not shown) indicated the two peaks with unconjugated mBBr. GSH and EGT were the only thiols in the wild type extracts. No EGT was detected in either the *NcΔEgt-1* conidia or mycelia.

weight thiols detected as mBBr derivatives in wild type conidia (Fig. 3B) and mycelia (Fig. 3E) eluted at the same time (12.5 and 29.3 min) as the GSH- and EGT-derivatized standards (Fig. 3A), respectively. Samples mixed with the derivatized standards coeluted with the standards (Fig. 3C). As in the HPLC-EC, the EGT concentration in the conidia (Fig. 3B) was much greater than in the mycelia (Fig. 3E). Consequently, the HPLC-fluorescence added a second line of evidence that the conidial-enriched peak identified in HPLC-EC was EGT. Compared to the GSH standards derivatized with mBBr, the same quantity of EGT derivatized with mBBr only produced 1.25% of the fluorescence signal, i.e., the relative response factor for the fluorescence detector at 394 excitation/490 emission nm for GSH/EGT was 80.

To conclusively identify the EGT in conidial lysates, *C. graminicola* conidial extracts and EGT standards were derivatized with mBBr, separated by reverse phase HPLC, collected at the time that

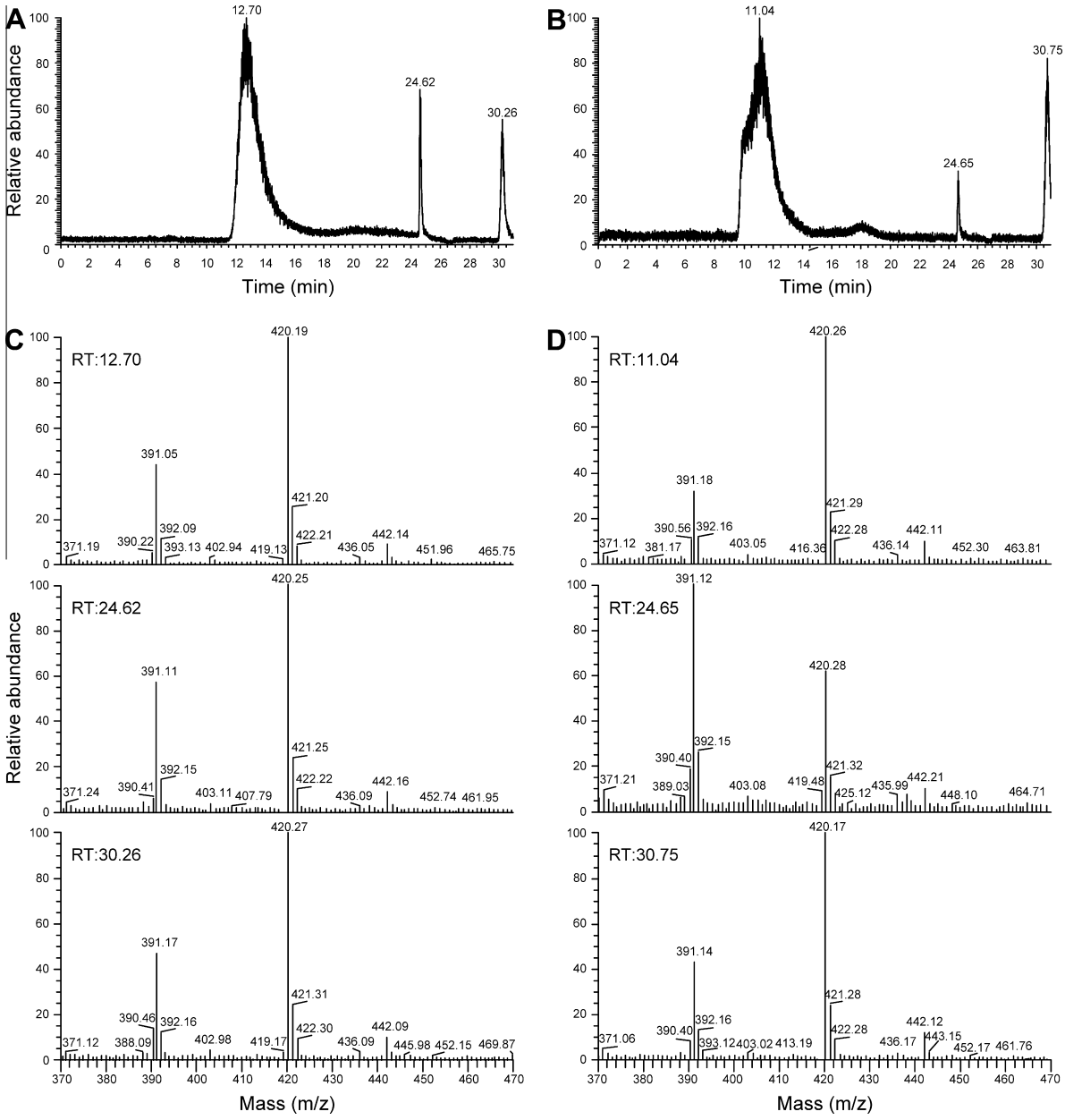

Fig. 4. Liquid chromatography (LC)-ESI positive ion mass spectrometric (MS) analysis of monobromobimane (mBBr) derivatives of an ergothioneine (EGT) standard (A, C) and the putative EGT-mBBr from *C. graminicola* conidia (B, D). (A, B) LC produced one major and two minor peaks with the expected mBBr-EGT mass of 420.5 $[M + H]^+$. (C, D) mass spectra of each peak from *m/z* 370–470. The mBBr-derivatized EGT standard and putative fungal EGT had indistinguishable elution profiles and mass spectra, with a major ion peak at 420.3.

the EGT-mBBr eluted, and analyzed by LC/MS in a positive ion mode. When the full LC/MS chromatograms of both the EGT-mBBr standard (Fig. 4A) and the putative conidial EGT-mBBr sample (Fig. 4B) were scanned with a *m/z* value of 420.5 $[M-H]^{+1}$ (corresponding to the expected mass of the positive ion of the EGT-mBBr derivative), both samples had one major peak and two minor peaks with nearly identical retention times. When the peaks from each retention time were examined for *m/z* values of 370–470, the standard (Fig. 4C) and sample (Fig. 4D) had similar spectra with the major ion *m/z* at 420.2. There also was a smaller peak at 391, which we postulate is a contaminant.

In *C. graminicola* and *N. crassa* conidia, the EGT concentration was significantly higher (~9-fold) than GSH (Table 1). While EGT concentrations were high in conidia (Figs. 2 and 3B), EGT concentrations were significantly lower in mycelia (Figs. 2D and 3E); EGT concentrations in mycelia were 6 and 20% of conidial concentration in *C. graminicola* and *N. crassa*, respectively (Table 1). We confirmed that there is no EGT in *S. cerevisiae* cells.

3.2. The gene NCU04343 is required for ergothioneine biosynthesis in a fungus

The *N. crassa* genome was searched for homologs of the enzymes in the *M. avium* EGT biosynthetic pathway. *N. crassa* has a putative γ-glutamyl cysteine synthase (NCU01157), but it only has weak ($E = 0.025$) homology to *M. avium* EgtA, a *N. crassa* knock-out is only available as a heterokaryon, and the knock out might be affected in glutathione biosynthesis. *N. crassa* knockouts in homologs of the *M. avium* EGT pathway were available in NCU04343, NCU01256, NCU07917, but not in NCU04636, the

putative EgtE homolog. Interestingly, bioinformatic analysis indicated that NCU04343 has domains in both EgtB and EgtD, consistent with Seebeck's (2010) observation of fusion proteins in fungal homologs of the *M. avium* EGT pathway.

To determine if any of the available knockout strains did not produce EGT, cell-free lysates of both wild type and knockouts of *N. crassa* conidia and mycelia were derivatized with mBBr, and the EGT-mBBr was quantified using HPLC with a fluorescence detector. Whereas the wild-type conidial and mycelial samples of *N. crassa* had EGT (Table 1), the NCU04343 knockout had no detectable EGT in either conidia (Fig. 3D) or mycelia (Fig. 3F). Knockouts in NCU01256, NCU07917 had wild type levels of EGT and GSH (data not shown) and consequently are not involved in EGT biosynthesis. Because NCU04343 is the first identified fungal gene required for EGT biosynthesis, we named it *NcEgt-1*.

3.3. Ergothioneine does not have discernible pleiotropic affects and does not appear to protect against either Cu^{2+} toxicity or superoxide in vivo

We tested the rate of hyphal extension of the wild type and *NcΔEgt-1* strains in both the nitrogen-rich and -deficient Vogel's medium in order to determine if the mutant was pleiotropically affected. Growth of both strains in both media was linear over time ($R^2 \geqslant 0.99$) (Fig. 5). A two-way ANOVA indicated no significant strain * media interaction ($P = 0.5$), a highly significant media effect ($P < 0.001$) and no strain effect ($P = 0.95$). That is, both wild type and *NcΔEgt-1* had similar growth in both Vogel's agar (4.2 ± 0.09 and 4.1 ± 0.2 mm h^{-1}, respectively) and in Vogel's without ammonium nitrate (2.4 ± 0.08 and 2.5 ± 0.09 mm h^{-1}, respectively).

Cupric sulfate was used to test if EGT protects against Cu^{2+} during conidial germination. A general linear model analysis shows that germination between the two strains in all the cupric sulfate concentrations was indistinguishable (Fig. 6); there was no significant strain * Cu^{2+} concentration interaction ($P = 0.8$), no significant strain effect ($P = 0.9$) and a highly significant Cu^{2+} concentration effect ($P < 0.0001$). Thus, endogenous EGT does not protect against cupric sulfate-toxicity during germination of *N. crassa* conidia.

We also tested whether EGT protects against Cu^{2+}-induced toxicity during mycelial growth. A general linear model analysis shows that mycelial growth of the two strains in Vogel's agar with and without 4.25 mM Cu^{2+} EGT was not significantly different between strains (Table 2); there was no significant strain * Cu^{2+} concentration interaction ($P = 0.81$), no significant strain effect ($P = 0.09$) and a highly significant Cu^{2+}concentration effect ($P < 0.0001$). Thus, endogenous EGT does not protect against cupric sulfate-toxicity during mycelial growth of *N. crassa*.

Menadione sodium bisulfate was used to test if endogenous EGT protects against superoxide during spore germination. Conidia of the *NcΔEgt-1* and wild type were incubated for 7.5 h in Vogel's agar amended with a range of menadione concentrations (Fig. 7). A general linear model analysis shows that there is no significant strain * menadione concentration interaction ($P = 0.53$), no significant strain effect ($P = 0.82$) and a highly significant menadione concentration effect ($P < 0.0001$). Thus, EGT does not protect against superoxide damage *in vivo* during *N. crassa* germination.

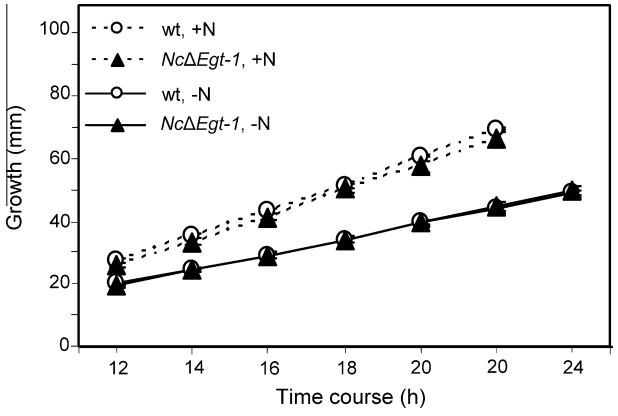

Fig. 5. Length of *N. crassa* wild type (○) and the *NcΔEgt-1* strain (▲) colonies grown in the dark on Vogel's with and without nitrogen. Data are the means ± SE of 3 replicates.

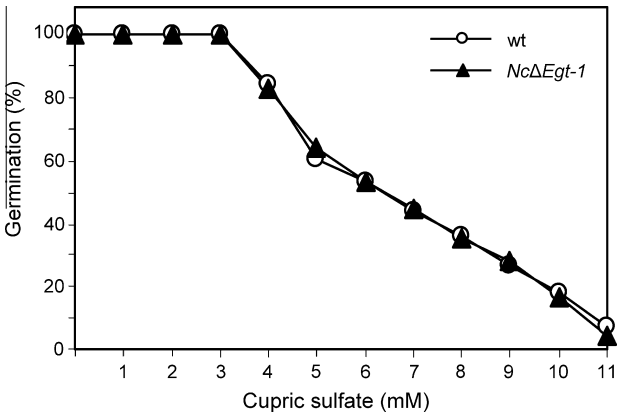

Fig. 6. Percentage germination of *N. crassa* wild type (○) and the *NcΔEgt-1* strain (▲) after 7.5 h in Vogel's agar amended with 0.01% Tween 20, 5 mM MES buffer (pH 6.1) and 0–11 mM cupric sulfate. Data are the means ± SE of 3 replicates.

Table 2
Dry weight of *Neurospora crassa* wild type and *NcΔEgt-1* mycelium produced in 20 h in Vogel's broth amended with 0.01% Tween 20, 5 mM MES buffer (pH 6.1) and either 0 or 4.25 mM cupric sulfate.

Strain	Cupric sulfate (mM)	Dry weight mycelium, mg ± SE[a]
Wild type	0	162.5 ± 9.9 a
NcΔEgt-1	0	144.3 ± 12.9 a
Wild type	4.25	53.2 ± 6.7 b
NcΔEgt-1	4.25	39.3 ± 4.8 b

[a] The analysis as a two-way ANOVA is described in the text. Data here are shown as a one-way ANOVA. Data are the means ± SE of 6 replicates. Means within a column followed by the same letter are not significantly different by Tukey's HSD ($\alpha = 0.05$).

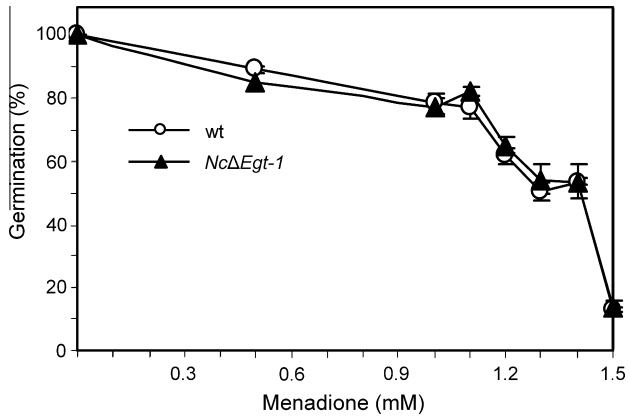

Fig. 7. Effect of the reactive oxygen-producing compound menadione on conidial germination of *N. crassa* wild type (○) and *NcΔEgt-1* strain (▲) incubated in Vogel's broth with 0.01% Tween 20. Percentage germination was assessed after 7.5 h in 0–1.5 mM menadione. Data are the means ± SE of 3 replicates.

3.4. Ergothioneine protects conidia against peroxide in vivo

Tert-butyl hydroperoxide was used to test the effect of peroxide on germination of *NcΔEgt-1* conidia in comparison to the wild type. Fig. 8A shows a time course of germination of the two strains in 0 and 4 mM *tert*-butyl hydroperoxide in Vogel's agar. In an analysis with a general linear model of transformed percentage germination over time in Vogel's agar without peroxide, there was no significant ($P = 0.97$) strain $*$ time interaction, no significant strain effect ($P = 0.9$) and a highly significant time effect ($P < 0.0001$). That is, in the absence of peroxide, both strains had an indistinguishable time course of germination. Based on the 95% confidence intervals, for all four strain-peroxide treatments, maximum germination occurred by 7.5 h. *NcΔEgt-1* was significantly more sensitive to 4 mM *tert*-butyl hydroperoxide than the wild type. After 7.5 h, percentage germination of *NcΔEgt-1* and wild type after 7.5 h incubation was 3 ± 1% and 68 ± 2%, respectively.

Fig. 8B shows the effect of a range of concentrations of *tert*-butyl hydroperoxide in Vogel's agar on percentage germination of the *NcΔEgt-1* and wild type conidia after 7.5 h incubation. For statistical analysis, the fraction germinated was logit-transformed. The logit fraction of *NcΔEgt-1* conidia germinated after 7.5 h = 3.6 − (1.3 × [*tert*-butyl hydroperoxide in mM]) and for wild type = 3.2 − (0.7 × [*tert*-butyl hydroperoxide in mM]). Analysis with a general linear model indicated that the concentration at which *NcΔEgt-1* germ tube emergence was affected was significantly lower ($P < 0.0001$) and the fraction germinated declined more rapidly with increasing concentration ($P < 0.0001$) than in the wild type. Based on the logit regression, germination of 50% (GI_{50}) of the *NcΔEgt-1* conidia was prevented at 2.7 mM *tert*-butyl hydroperoxide whereas the GI_{50} for the wild type was 4.7 mM *tert*-butyl hydroperoxide, or at a 1.7X greater concentration. Consequently, endogenous EGT provides protection against peroxide damage during *N. crassa* germination.

When conidia were incubated in Vogel's agar with 7 mM *tert*-butyl hydroperoxide for 40 min, and then stained with the reactive oxygen species indicator carboxy-H_2DCFDA for an additional 40 min, 68 ± 4% of the *NcΔEgt-1* conidia fluoresced in contrast to 10 ± 2% of the wild type ($P = 0.0002$). None of the wild type or *NcΔEgt-1* conidia fluoresced when incubated in media without *tert*-butyl hydroperoxide. Although the light used to capture the images shown in Fig. 9 induces carboxy-H_2DCFDA fluorescence, the qualitative differences in the four treatments are clear: (1) in the presence of exogenous *tert*-butyl hydroperoxide, *NcΔEgt-1* conidia produce more reactive oxygen species than the wild type; and (2) in the absence of *tert*-butyl hydroperoxide, there are no apparent differences in reactive oxygen species in *NcΔEgt-1* conidia versus the wild type cells. Thus, endogenous EGT quenches peroxide.

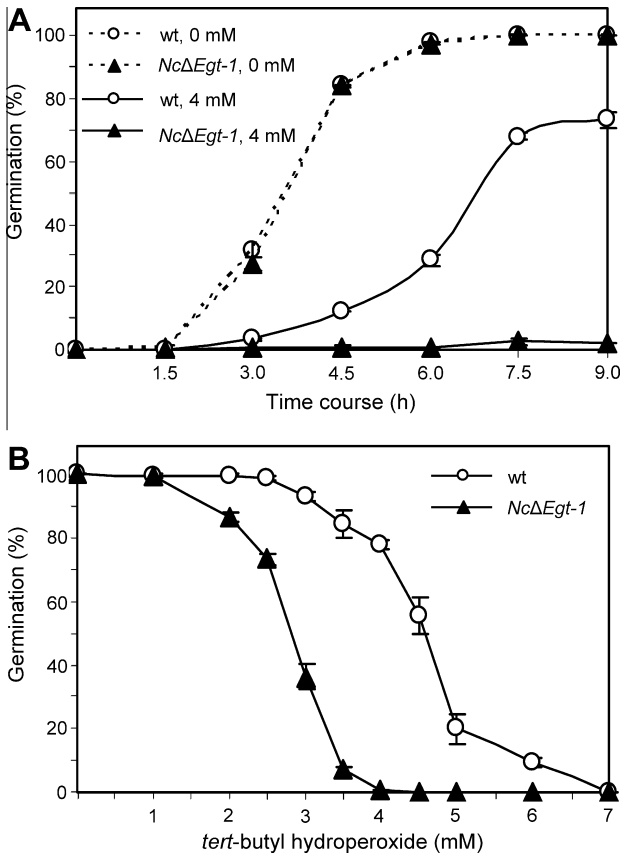

Fig. 8. Effect of the reactive oxygen-producing compound *tert*-butyl hydroperoxide on conidial germination of the *N. crassa* wild type (○) and the *NcΔEgt-1* strain (▲) incubated in Vogel's broth with 0.01% Tween 20. Data are the means ± SE of 3 replicates. A) Percentage germination time course with 0 and 4 mM *tert*-butyl hydroperoxide. B) Percentage germination after 7.5 h in 0–7 mM *tert*-butyl hydroperoxide.

3.5. Ergothioneine has a protective role in conidial longevity

As shown in the 0 mM controls in Figs. 6–8B, five to 10-day old conidia from both *NcΔEgt-1* and the wild type germinated 100 ± 0% in Vogel's medium. Preliminary tests indicated that germination is independent of genotype when conidia are harvested from cultures that are 21 or less days old. Consequently, conidia removed from 17 to 84-day-old slants were tested to determine if conidial longevity is affected by endogenous EGT (Fig. 10). A general linear model analysis of the log-transformed percentage germination data as a function of conidial age indicated a highly significant difference in slopes in the regression lines ($P_{strain*age} < 0.001$), i.e., wild type conidia remained viable for significantly longer than *NcΔEgt-1* conidia. The effects of conidial age ($P < 0.001$) and strain ($P < 0.001$) were also highly significant. Based on the linear regressions ($R^2 = 0.93$ and 0.89 for wild type and *NcΔEgt-1*, respectively) with log (% $\text{germinated}_{\text{wild type}} + 1$) = 2.26 − (0.016 × conidial age in days) and log (% $\text{germinated}_{NcΔEgt\text{-}1} + 1$) = 2.36 − (0.027 × conidial age in days), the time to non-germinability in Vogel's agar of 50% of the conidia is 35.1 days and 24.6 days for wild type and *NcΔEgt-1*, respectively, which is equivalent to a 30% reduction in conidial life span in the *NcEgt-1* deletion strain.

3.6. Distribution of NcEgt-1 homologs in fungi

In silico searches for NcEgt-1 homologs were performed in fungal species in all taxa in which there are full genome sequences available (Supplemental Table 1). NcEgt-1 homologs are present in all of the true fungal groups in which EGT has been identified: Mucoromycotina (i.e., Mucorales), Agaricomycotina (i.e., Agaricales, Boletales, and Cantharellales), Pucciniomycotina (i.e., Sporidiobolales), and the Pezizomycotina (i.e., Eurotiales, Glomerellales, Hypocreales, and Sordariales) (Dubost et al., 2007; Ey et al., 2007; Genghof, 1970; Melville et al., 1956; this report). Genghof (1970) reported that the slime mold *Physarum polycephalum* (Amoebozoa) has EGT, but sequence is not yet available. All analyzed NcEgt-1 homologs have the same domains as NcEgt-1 with an overall identity from 47% to 95% in *Ustilago maydis* and *Sordaria macrospora*, respectively. Notably, members of the Saccharomycotina in the Ascomycota tested for EGT (*S. cerevisiae, Pichia* spp., and *Candida albicans*) do not have EGT (Ey et al., 2007; Genghof, 1970; this report) and do not have a NcEgt-1 homolog. No NcEgt-1 homologs were found in sequenced genomes of Microsporidia, which

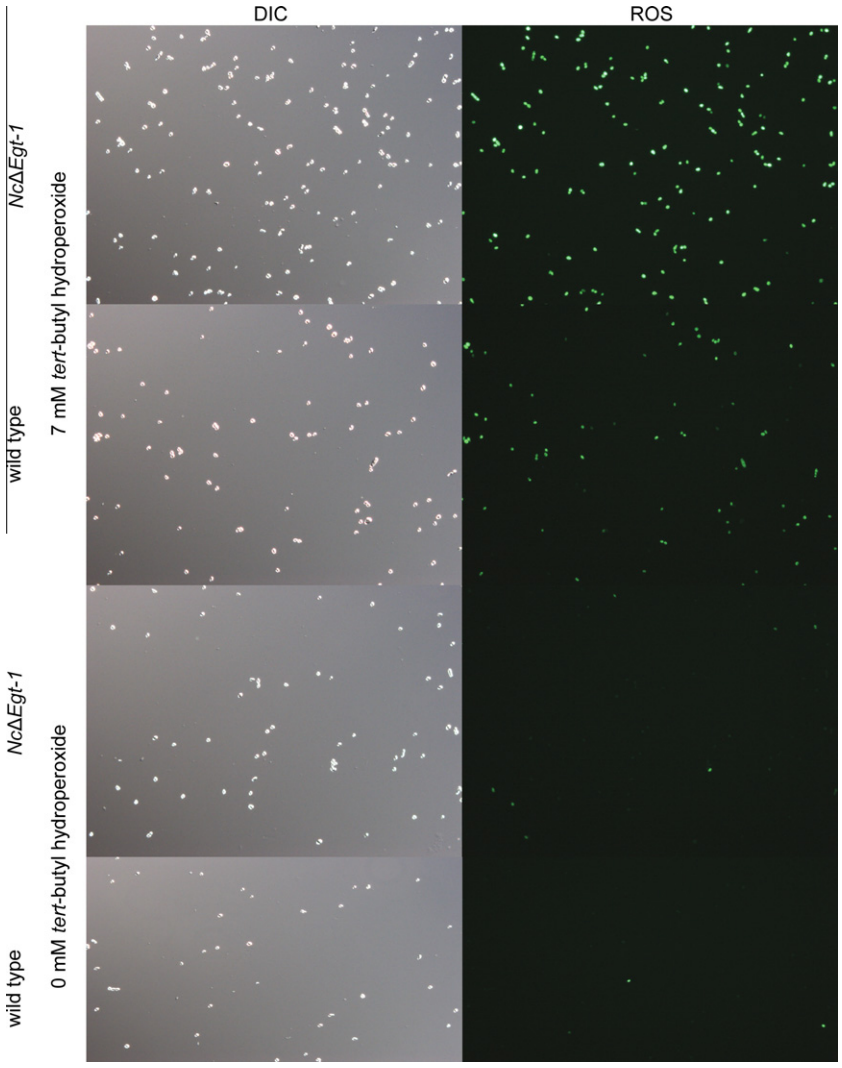

Fig. 9. Effect of 0 and 7 mM *tert*-butyl hydroperoxide on production of fluorescent reactive oxygen species (ROS) produced by 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate in conidia of the *N. crassa* wild type and *NcΔEgt-1* strain. All epifluorescent ROS images were taken using a green fluorescent protein (GFP) filter cube and the same camera settings. When exposed to *tert*-butyl hydroperoxide, there was greater ROS production in *NcΔEgt-1* than in wild type conidia. In the absence of *tert*-butyl hydroperoxide, no differences between the mutant and the wild type were observed.

may be the basal lineage of fungi (Corradi and Slamovits, 2011). Similarly, we found no EGT in sporangia of the biotrophic oomycete (Stramenopiles) *Bremia lactucae* (data not shown).

4. Discussion

4.1. Ergothioneine is an antioxidant against peroxide in conidia and contributes to conidial longevity

We demonstrated that a knockout strain in the NCU04343 gene, *NcΔEgt-1*, does not produce EGT in conidia or mycelia, in contrast to the wild type. The *NcΔEgt-1* strain is not pleiotropically affected; hyphal elongation was similar for both the wild type and *NcΔEgt-1* strains in both Vogels's agar with and without nitrogen. In addition, the superoxide-producer menadione had indistinguishable effects on conidial germination between the two strains. Cupric sulfate also had indistinguishable effects on conidial germination and on hyphal growth between the two strains.

Utilization of an EGT-minus mutant allowed us to demonstrate that endogenous EGT in conidia can quench exogenous peroxide. Germination of *NcΔEgt-1* conidia was significantly more sensitive to *tert*-butyl hydroperoxide than the wild type. When incubated for 40 min in *tert*-butyl hydroperoxide, the wild type had significantly ($P = 0.0002$) fewer conidia with a visible quantity of the reactive oxygen species indicator carboxy-H_2DCFDA than the *NcΔEgt-1*. Thus, endogenous EGT quenches peroxide and serves as antioxidant *in vivo* in conidia. Interestingly, *Schizosaccharomyces pombe* microarray data shows that the *NcEgt-1* homolog is up-regulated 10-fold when cells are exposed to H_2O_2 (Chen et al., 2003). Paul and Snyder (2010) examined the role of EGT in human HeLa cells, which normally express the EGT-transporter OCTN1 and consequently concentrate EGT, using RNA interference; they demonstrated that endogenous EGT protected HeLa cells from the toxicity of exogenous H_2O_2 and from peroxide-induced degradation of mitochondrial DNA. Somewhat similarly, EGT decreased the mutagenic effects of *tert*-butyl hydroperoxide in *Salmonella* cells (Hartman and Hartman, 1987). However, two previous studies using other cells lines and experimental strategies concluded that EGT is not an effective antioxidant of peroxides (Aruoma et al., 1999; Ey et al., 2007). Similarly, various effects of the addition of EGT either to cells or *in vitro* in the presence of peroxides have been reported. Franzoni et al. (2006) concluded that EGT was the most active scavenger against three species of free radicals

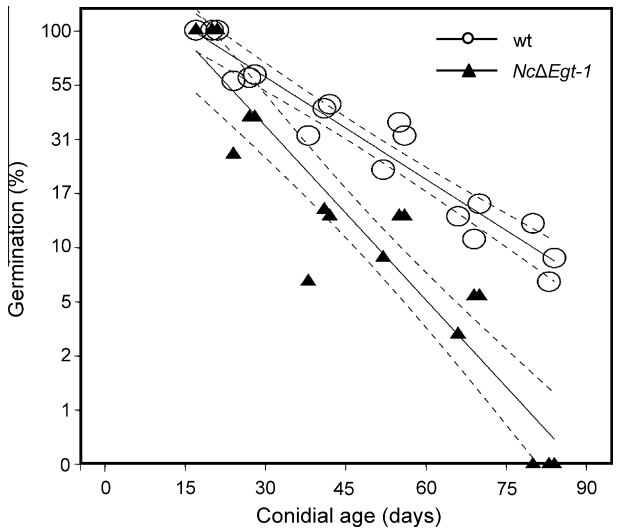

Fig. 10. Percentage germination after 7.5 h in Vogel's broth of 17–84-day-old conidia of the *N. crassa* wild type (○) and the *NcΔEgt-1* strain (▲). Germination data were log (percentage germination + 1) transformed to linearize the data (R^2 = 0.93 and 0.89 for the wild type and *NcΔEgt-1*, respectively); the *y* axis is on a log scale. The solid regression lines and dotted 95% confidence intervals are shown. A general linear model analysis showed that *NcΔEgt-1* conidia have a highly significantly reduced life span ($P_{\text{strain}*\text{age interaction term}} < 0.001$) compared to the wild type.

(peroxyl, hydroxyl, and peroxynitrite) compared to GSH, Trolox and uric acid. In contrast, Akanmu et al. (1991) concluded that EGT at the physiological concentration in liver cells did not prevent lipid peroxidation of liver microsomes.

Although EGT provided some protection against peroxide-induced toxicity to conidial germination, EGT did not protect *N. crassa* conidia against toxicity from menadione, a superoxide producer. Rose bengal, which generates superoxides in the light, similarly did not have a differential effect on the two *N. crassa* strains; conidia of both strains in 12 μM rose bengal in Vogel's had indistinguishably suppressed germination in the light (data not shown). In contrast, Paul and Snyder (2010) showed that a pre-treatment of EGT protected HeLa cells from growth-suppression and apoptosis of the superoxide producer pyrogallol. However, in *in vitro* studies, EGT did not quench superoxide (Dubost et al., 2007; Hartman et al., 1988). Indeed, Hartman et al. (1988) demonstrated that when EGT instead of methionine was added as a reducing agent, superoxide formation was increased approximately 25-fold.

Cupric sulfate also had indistinguishable effects on conidial germination and on hyphal growth between the two strains. In contrast, Ey et al. (2007), showed that human embryonic kidney (HEK-293) cells transfected with the OCTN1 vector that were pre-incubated with EGT before Cu^{2+} exposure had less copper toxicity than cells transfected with the empty vector. Nonetheless, consistent with our results, they concluded that EGT would have little effect on Cu^{2+} toxicity *in vivo*.

Although five to 21-day-old conidia of both strains germinated 100%, *NcΔEgt-1* conidia had significantly ($P < 0.001$) diminished longevity, with a 29% reduction in conidial life span in the *NcEgt-1* deletion strain. The fact that EGT has a function in the life cycle between conidiogenesis and conidial germination is consistent with our observation that EGT concentrations in *N. crassa* are 5-fold higher in conidia than in mycelia. Conidia in nature are exposed to environmental conditions, including desiccation and light, that produce reactive oxygen species (ROS). Consequently, we propose that EGT protects conidia from auto-produced ROS. Aguirre et al., 2005 have postulated that ROS actually initiate cell differentiation in fungi, and that ROS is required for sporulation (Belozerskaya and Gessler, 2006). At the beginning of conidiogenesis, *N. crassa* has a transient hyperoxidant state that coincides with a decrease in GSH and an increase in the oxidation of GSH and NADPH (Toledo et al., 1995). Similarly, during the onset of conidial germination, there is an increase in intra-cellular singlet oxygen, which results in protein oxidation (Lledias et al., 1999). Thus we postulate that EGT has a role in redox reactions, particularly in fungal spores.

4.2. Identification, evolution, and function of NcEgt-1

We identified the first EGT biosynthetic gene in a filamentous fungus, *N. crassa* (NCU04343), based on amino acid sequence of genes involved in EGT biosynthesis in the actinomycete *M. smegmatis*. NcEgt-1 is a multidomain protein: it has a N-terminal SAM-dependent methyltransferase with homology to *M. smegmatis* EgtD (27% identity); and a C-terminal region with a DinB_2 and a FGE-sulfatase with homology to *M. smegmatis* EgtB (24% identity) (Marchler-Bauer et al., 2009; Seebeck, 2010). EgtD and EgtB homologs are present in some members of diverse bacterial taxa: the actinobacteria, cyanobacteria, acidobacteria, firmicuites, and proteobacteria (Supplemental Table 2). In addition, EgtD and EgtB homologs are adjacent genes in multiple prokaryotes. Consequently, we postulate that the fungal gene is derived from an ancient fusion of two genes of prokaryotic origin.

Recently, *M. smegmatis* EgtD and EgtB were biochemically characterized (Seebeck, 2010). EgtD is a histidine-specific methyltransferase (Seebeck, 2010), consistent with the *N. crassa* EGT pathway from histidine to the trimethylated hercynine (Askari and Melville, 1962; Melville et al., 1959). The *M. smegmatis* EgtB catalyzes the iron(II)-dependent oxidative sulfuration of hercynine *in vitro*, consistent with the *N. crassa* pathway from hercynine to hercynylcysteine sulfoxide (synonym, *S*-(β-amino-β-carboxyethyl)ergothioneine sulfoxide) (Ishikawa et al., 1974). NcEgt-1 also contains the iron binding motif (HX_3HXE) in the Din_B2 domain, which is also present in both *M. smegatis* EgtB (Seebeck, 2010) and in *Trypanosoma cruzi* OvoA (Braunshausen and Seebeck, 2011). *T. cruzi* OvoA catalyzes an intermediate step in biosynthesis of another histidine derivative, ovothiol from histidine to 5-histidylcysteine sulfoxide. Similar to NcEgt-1, OvoA has three domains: a methyltransferase domain, a FGE-sulfatase domain (with 24% identity to NcEgt-1 FGE-sulfatase domain), and a Din_B2 domain. Amino acid substitutions (i.e., either of two histidines or glutamic acid) in the HX_3HXE motif in the Din_B2 domain in the N-terminus of OvoA reduced its enzymatic activity by at least 100-fold (Braunshausen and Seebeck, 2011). Because we did not detect any impact on EGT biosynthesis in the knockout of the other potential methyltransferase (NCU07917) and because NCU04343 is the best candidate for the DinB_2/FGE-sulfatase, we propose that NcEgt-1 is a bifunctional enzyme that catalyzes the first two steps of EGT biosynthesis from histidine to hercynine and then to hercynylcysteine sulfoxide (Fig. 1A), consistent with the previously proposed biosynthetic pathway of EGT in *N. crassa* (Ishikawa et al., 1974). The gene that encodes for the enzyme that catalyzes the conversion of hercynylcysteine sulfoxide to EGT remains unidentified. *N. crassa* has a single gene (NCU04636) that putatively encodes for a cysteine desulfurase and has 34% identity with *M. avium* EgtE; this *N. crassa* knockout has not been tested for EGT.

The presence of EGT in many of the taxa with *NcEgt-1* homologs remains to be determined biochemically (Supplemental Table 1). For example, *Schizosaccharomyces. pombe* in the Taphrinomycotina has a NcEgt-1 homolog. However, *Sc. pombe* does not have EGT in its vegetative cells (data not shown); however, conditions (peroxide-exposure and meiosis) in which the NcEgt-1 homolog is upregulated were not examined (Chen et al., 2003; Mata et al., 2002). Of the 30 surveyed genomes with NcEgt-1 homologs, 17%

and 23% of the methyltransferase and FGE-sulfatase domains are split, and 10% have splits in both domains. Both *C. graminicola* and *N. crassa*, which produce EGT, have an intact methyltransferase and a split FGE-sulfatase domain; it is unknown whether the splits in either the methyltransferase or the FGE-sulfatase have any impact on protein function.

4.3. Detection, quantification, and dynamics of ergothioneine

This work is the first to assess concentrations of EGT in both conidia and mycelia in the same HPLC assay. Using mBBr derivatives and fluorescent detection, the data show that EGT is in both conidia and hyphae of *C. graminicola* and *N. crassa*, and that the concentrations in conidia are significantly greater than in mycelia with approximately 17- and 5-fold greater in conidia of *C. graminicola* and *N. crassa*, respectively, than in mycelia. Previously, Fahey et al. (1980) detected both GSH and EGT in *N. crassa* conidia, but did not quantify the concentrations. In both *C. graminicola* and *N. crassa* conidia, EGT is present in a higher concentration than GSH. EGT is concentrated in certain animal tissues; in bovine eye lenses, EGT is in approximately 10-fold higher concentration than GSH (Shires et al., 1997). The localization of both NcEgt-1 and EGT is unknown; the homolog of NcEgt-1 in *Schizosaccharomyces pombe* (SPBC1604.01) apparently is present in both the nucleus and the cytoplasm (Matsuyama et al., 2006). The data shown here indicate that EGT concentrations in the conidia in the two fungi differ with 25.4 ± 1.3 nmol EGT mg^{-1} protein (equivalent to 1841 ± 94 ng EGT mg^{-1} dry weight) in *N. crassa* and 11.1 ± 0.9 nmol EGT mg^{-1} protein (equivalent to 573 ± 44 ng EGT mg^{-1} dry weight) in *C. graminicola*. However, differences in media and culture conditions might affect EGT concentrations. Nonetheless, the data confirm earlier reports that show a range of EGT concentrations in different fungi.

With a significantly higher concentration of EGT in conidia than in mycelia, the data are consistent with the hypothesis that EGT biosynthesis is induced during sporulation. In *N. crassa*, conidiation is light-induced. In a genome-wide transcriptional analysis, *NcEgt-1* was annotated as a light-induced gene (Chen et al., 2009). Similarly, ascosporogenesis may also induce EGT biosynthesis. The *Sc. pombe NcEgt-1* homolog (mug158/SPBC1604.01) is up-regulated (~10-fold) during ascospore development, which involves meiosis. Consequently, mug158 was annotated as a meiotically up-regulated gene (Mata et al., 2002). Overall, the up-regulation of mRNA expression of *NcEgt-1* homologs during conidiation and the increased concentration of EGT in conidia suggests that EGT may have a role in protection of mitospores and meiospores from oxidative stress.

At the presumed cytoplasmic pH of 7.5 in fungi (Bagar et al., 2009), EGT is almost completely a thione rather a thiol (see Fig. 1A). At higher pHs, EGT ionizes to the thiolate form (Hand and Honek, 2005). Because of the predominance of the thione rather than the thiol, EGT is more stable in neutral aqueous solution than GSH and consequently more resistant to auto-oxidation (Hand and Honek, 2005). Using an electrochemical detection system of non-derivatized EGT and GSH standards (Lakritz et al., 1997), the GSH response factor relative to EGT (integrated area $pmol^{-1}$ GSH standard/integrated area $pmol^{-1}$ EGT standard) was ~50. This is consistent with EGT being less susceptible to auto-oxidation than GSH.

To our knowledge, this is the first use of bimane derivatives to identify EGT by LC/MS, and to quantify EGT by HPLC-EC. Although the limits of detection of GSH by either HPLC-EC or the mBBr derivatives with a fluorometric detector are similar (1 pmol GSH), HPLC-EC is a less sensitive method of EGT quantification (0.1 nmol EGT) than HPLC-fluorometry with the mBBr derivatives (13 pmol EGT). In addition, the mBBr-derivative peaks are well separated, in contrast to the HPLC-EC. That is, even though mBBr-EGT derivatives have a low fluorescent yield (Fahey et al., 1981), the sensitivity of detection of mBBr-EGT was excellent and equal to or greater than those in early studies.

The fluorescence response factor (integrated area $pmol^{-1}$ mBBr-GSH standard/integrated area $pmol^{-1}$ mBBr-EGT standard) was 80 ± 7. Previously, using extracts derivatized with either mBBr or monobromotrimethylammoniobimane (qBBr) followed by separation by cation-exchange chromatography, Fahey et al. (1981) reported a GSH fluorescence response factor relative to EGT of 10 and 40, respectively. Newton et al. (1981) reported a GSH to EGT fluorescence response factor of 12.5. The discrepancy between the different studies may be due to conditions of sample preparation and separation, and the fluorometry equipment (Fahey and Newton, 1987).

When both the mBBr-EGT standard and the eluate from the HPLC that corresponded to the mBBr-EGT peak were analyzed in LC/MS, both samples eluted as three major peaks, at the same three retention times. When each peak was scanned for the calculated *m/z* of 420.5, all peaks had very similar ion profiles. Consequently, both the mBBr-EGT standard and sample have an identical MS "fingerprint." In addition, EGT appears to form three distinct mBBr-EGT adducts.

To conclude, EGT was identified as the major low molecular weight thiol in conidia of the ascomycetes *C. graminicola* and *N. crassa*; it was identified by HPLC-EC, HPLC-fluorescence of mBBr-EGT conjugates, and, most authoritatively, by LC/MS of mBBr-EGT conjugates. Picomoles of EGT were quantified from small amounts of fungal tissue using a simple extraction procedure. Mutational analysis indicates that endogenous EGT helps protect conidia during the quiescent period between conidiogenesis and germination. The data further suggest that EGT helps protect conidia during the germination process from the toxicity of peroxide but not from superoxide or Cu^{2+}. Finally, the data are consistent with the hypothesis that endogenous EGT has more impact on conidial survival and germination than on hyphal growth.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fgb.2011.12.007.

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