γ-Glutamyltransferases (GGT) in Colletotrichum graminicola: mRNA and enzyme activity, and evidence that CgGGT1 allows glutathione utilization during nitrogen deficiency

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1. Introduction

Glutathione (GSH; L-γ-glutamyl-L-cysteinyl-glycine) is present in 3–10 mM concentrations in eukaryotic cells and can account for up to 1% of the dry weight of Saccharomyces cerevisiae (Meister and Anderson, 1983; Pócsi et al., 2004). GSH plays a role in thiol-redox maintenance (Penniclnx and Elskens, 1993; Pócsi et al., 2004) and in cytosolic iron metabolism (Kumar et al., 2011). Notable features of GSH include its unusual γ-glutamyl peptide bond, which makes it resistant to proteolytic degradation by most peptidases (Penniclnx and Elskens, 1993; Pócsi et al., 2004). Although the GSH biosynthesis pathway is relatively well studied, less is known about GSH degradation in fungi. S. cerevisiae's only γ-glutamyltransferase (GGT; EC 2.3.2.2, synonym, γ-glutamyl-transpeptidase), is encoded by the ORF ECM38/CIS2/YLR299w (Jaspers and Penniclnx, 1984; Jaspers et al., 1985; Mehdi et al., 2001). GGT catalyzes the transfer (transpeptidation) of the γ-glutamyl moiety from GSH and γ-glutamyl compounds to amino acids, to GSH itself, or to water as a part of hydrolysis (Penniclnx and Elskens, 1993). Recently, Kaur et al. (2012) elucidated an additional glutathione degradation pathway (DUG) in S. cerevisiae in which Dug2p and Dug3p form an amidotransferase that cleaves the γ-glutamyl bond. A dipeptidase Dug1p then cleaves the cysteinyl-glycine (Kaur et al., 2009).

S. cerevisiae with a knockout in the CIS2 gene is viable (Lussier et al., 1997; Mehdi et al., 2001). In Schizosaccharomyces pombe, which has two GGTs, the ggt1Δ is lethal, but strains with ggt2Δ are viable (Kim et al., 2010). Both S. cerevisiae Cis2p and S. pombe Ggt1 are localized in the vacuolar membrane, whereas the S. pombe
GGTs in *S. pombe* appear to be involved in the response to several types of oxidative stress. *ggt2* transcripts are induced by H$_2$O$_2$, and *ggt1* and *ggt2* transcripts are induced by sodium nitroprusside (Kang et al., 2005; Park et al., 2004). In *S. cerevisiae*, deletion of the CIS2 gene rendered the yeast cells more sensitive than the wild type to 5 mM H$_2$O$_2$ (Springael and Penninckx, 2003); however, transcription of *CIS2* is not induced by 1 mM H$_2$O$_2$ (Springael and Penninckx, 2003). Similar to *S. cerevisiae*, a *GGT1* knockout strain of *Hansenula polymorpha*, which had approximately 35–40% of the wild type GGT activity, was also more sensitive to 0.8 mM tert-butyl hydroperoxide than the wild type (Ubayovk et al., 2006). Park and associates (2004) showed that a *S. pombe* strain over-expressing endogenous *ggt1* grew in media containing 3 mM H$_2$O$_2$, in contrast to the wild type. However, a strain over-expressing *ggt2* had only slightly greater survival on media with 4 mM H$_2$O$_2$ compared with the wild type (Park et al., 2005). Gales et al. (2008) showed that a CIS2A mutant of *S. cerevisiae* tolerated more H$_2$O$_2$ than the wild type. The conflicting results of the last two papers with the previous studies may be due to the use of different assays for H$_2$O$_2$ toxicity and different yeast strains. Kumar et al. (2003a) demonstrated that some widely used strains of *S. cerevisiae* have polymorphisms in the CIS2 locus, both in the promoter and coding region, that result in “wild type” phenotypes with no GGT activity.

Several lines of evidence indicate that GSH also can serve as a nutrient source during nitrogen and sulfur starvation in *S. cerevisiae* (Elskens et al., 1991; Kumar et al., 2003b; Mehdī and Penninckx, 1997) and in *S. pombe* (Song and Lim, 2008). GSH and GGT activity, increased during nitrogen starvation in *S. cerevisiae* (Mehdī and Penninckx, 1997) and *S. pombe* (Song and Lim, 2008). In *S. cerevisiae*, more than 90% of the GSH is mobilized to the central vacuole in nitrogen-starved cells (Mehdī and Penninckx, 1997). Sulfur deprivation and GSH deficiency also increased GGT activity in *S. cerevisiae* (Elskens et al., 1991). Expression of yeast GGTs also depends on the nitrogen source. GGT activity in *S. cerevisiae* is repressed by ammonium but is induced by urea, glutamate and proline (Jaspers et al., 1985; Springael and Penninckx, 2003). The repression of *S. cerevisiae* GGT by ammonium occurs at the transcriptional level (Springael and Penninckx, 2003). During nitrogen starvation, transcription of *S. pombe* *ggt2* but not *ggt1* is dependent on the transcription factor Pap1 (Kim et al., 2005; Song and Lim, 2008).

In *S. pombe*, the two GGT genes are differentially regulated by carbon sources and metabolic stress. Non-fermentable carbon sources such as acetate and ethanol increase transcript levels of *S. pombe ggt1* (Kim et al., 2005), although glycerol induces transcription of *ggt1* and *ggt2* (Kang et al., 2005; Park et al., 2004). In contrast, fermentable carbon sources (i.e., glucose at low concentrations, lactate, and sucrose) increase transcription levels of *ggt2* but not of *ggt1* (Kang et al., 2005; Kim et al., 2005).

Based on predicted amino acid sequence, there are three well-supported main clades of GGTs in the Ascomycota (*Bello and Epstein, 2013*). One clade has the three relatively well-characterized fungal GGTs (*S. cerevisiae* CIS2, and *S. pombe* GGT1 and GGT2), and only contains GGTs in the Saccharomycotina and Taphrinomycotina. Most of the GGTs in this clade have all 14 of the highly conserved and critical amino acids in GGTs in the other kingdoms. A second clade is a Pezizomycotina-only clade; this clade differs in two of the 14 highly conserved amino acids found in GGTs in the other kingdoms and contains Colletotrichum graminicola (teleomorph, Glomerella graminicola, a Sordariomycete in the Pezizomycotina in the Ascomycota) CgGGT1 and CgGGT2. A third clade (GGT3) differs in 11 of the 14 highly conserved amino acids in GGTs in the other kingdoms and contains *C. graminicola* CgGGT3. Because there has been an expansion of GGTs in the Pezizomycotina compared to the yeast clades in the Ascomycota, we investigated GGTs in *C. graminicola*. In this study we show that CgGGT1 is expressed at a higher level than the other two CgGGTs. Examination of strains lacking or over-expressing CgGGT1, which had significantly lower and higher GGT activity than the wild type, respectively, suggests that CgGGT1 is involved in utilization of GSH during nitrogen deficiency in vitro. Although the strains had differing levels of GGT, they all had the same level of GSH in vitro, and were as virulent as the wild type in wounded corn leaves.

2. Materials and methods

2.1. *Colletotrichum graminicola* strains and culture conditions

*C. graminicola* strains were stored and falcate conidia were produced as indicated in Bello et al. (2012). Mycelia were produced as described (Bello et al., 2012) except that flasks with 40 ml of Fries broth were incubated at 28 °C and collected onto Miracloth (Calbiochem, San Diego, CA, USA). Germlings were produced in modified Fries (Fries with 1% w/v glucose instead of sucrose and with 0.01% Tween 20) either with or without nitrogen (i.e., without ammonium tartrate, ammonium nitrate, and yeast extract). To produce germlings, washed conidia (1.5 × 10⁶) were suspended in 5 ml modified Fries in 100-mm diameter (diam) polystyrene dishes, and incubated stationary in the dark at 30 °C. After 12 h, media was poured-off, plates were rinsed with water to remove ungerminated conidia, 1 ml water was added, and germlings were dislodged with a rubber cell scraper and transferred to ice-cold tubes. Eight and 30 plates were collected for germlings produced with and without nitrogen, respectively, per replicate. When indicated, tubes containing fungal tissues were flash-frozen and lyophilized.

2.2. Molecular methods

2.2.1. Cell breakage and isolation of DNA and RNA

Ten mg of lyophilized conidia were ground manually with a metal pestle in a 1.5 ml microfuge tube with 250 μl of 0.5 mm diam glass beads (BioSpec, Bartlesville, OK, USA). Genomic DNA was isolated using the DNeasy Plant Mini kit (Qiagen, Valencia, CA, USA).

Total RNA was isolated using the RNasy Plant Mini kit (Qiagen). Ten mg of lyophilized conidia or mycelia were ground as above. Lyophilized germlings (Section 2.1) were disrupted in 500 μl lysis buffer with 250 μl of 0.5 mm diam glass beads in a FastPrep system (BioSpec) with 8 cycles of shaking and 30 s of ice-cooling. Unwounded corn leaf sheaths of Mo940 were mock-inoculated or inoculated with the wild type using a 4-mm diam needle. Sheath discs on dry-ice were ground in liquid nitrogen with a mortar and pestle. Purified RNA was treated with the TURBO DNA-free kit (Ambion, Austin, TX, USA). The integrity and yield of both DNA and RNA were determined by gel electrophoresis and spectrophotometric analysis using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA).

2.2.2. Standard PCR for cloning and sequencing

Primers were designed using Vector NTI v11.5 (Invitrogen, Carlsbad, CA, USA). Except when indicated, PCR reactions (50 μl) contained 1X GoTaq PCR Mastermix (Promega, Madison, WI,
USA) with 1.25 U Taq DNA polymerase, 1.5 mM MgCl2, 200 μM each of the dNTPs, 0.4 μM of each primer, and 10–50 ng of genomic or 1–5 ng of plasmid DNA as template in a MyCycler (Bio-Rad, Hercules, CA, USA). The annealing temperature for each primer pair was optimized.

For cloning and sequencing, QIAquick PCR purification kits (Qiagen), FastDigest restriction endonucleases (Fermentas) and T4 DNA ligase (Fermentas) were used. One Shot TOP10 Escherichia coli (Invitrogen) were transformed with plasmids by electroporation with a Gene Pulser Xcell System (Bio-Rad). Plasmid DNA was purified using a QIAprep Miniprep kit (Qiagen). Nucleotide sequences were determined with an ABI 3730 DNA sequencer (Applied Biosystems, Foster City, CA, USA) and sequences were analyzed with Vector NTI.

2.2.3. “Conventional” and quantitative reverse transcriptase PCR (RT-PCR)

For two-step RT-PCR reactions, cDNA was synthesized from 1.5 μg total RNA using MMLV reverse transcriptase (Promega) with an oligo(dT)20 primer. Negative controls included DNase-treated genomic DNA and samples that were not treated with reverse-transcriptase. cDNA was amplified with GGT1 seq4 primers, which amplifies 602 bp of the CgGGT1 gene from the wild type but not from the knockouts (Fig. 1A). β-tubulin (TUB2)-specific primers (Itub2 RT), which amplify 829 bp across an exon–exon junction, were used as an internal control. PCR reactions were performed as described above (Section 2.2.2).

The quantitative RT-PCR (qRT-PCR) primers (Table 1) produce 142–161 bp amplicons. Primers for CgGGT2 span an exon–exon junction. PCR reaction mixtures (20 μl) contained 5 μl of a 1:10 dilution of cDNA, 1X Fast SYBR Green Master Mix (Applied Biosystems), and 0.2 μM of each primer. The 7500 Fast Real-Time PCR System (Applied Biosystems) was set at 95 °C for 20 s followed by 40 cycles at 95 °C for 3 s and 60 °C for 30 s. Amplification specificity was confirmed by the expected dissociation curve and gel electrophoresis analysis. Fluorescence thresholds were set manually at 0.05. The amplification efficiency for each target and reference was determined in three independent trials by PCR using serial dilutions of cDNA as template. An ANOVA of amplification efficiency supported normalization of CgGGT1 (94.7% ± 1.4% efficiency, ±SE) with CgTUB2–5 (94.8% ± 0.3%), and normalization of CgGGT2 (98.1% ± 4.4%) and CgGGT3 (97.9% ± 1.3%) with CgTUB2–6 (100.5% ± 0.5%). Differences in gene expression between isolates were calculated using the 2–ΔΔCt method (Livak and Schmittgen, 2001). Each qRT-PCR reaction was performed in a randomized complete block design with one replicate per treatment on either three (germings grown in either modified Fries medium with or without nitrogen) or four (mycelia and inoculated leaves) different trial dates, respectively.

2.2.4. Cloning of CgGGT1

The amino acid sequence of the single GGT in S. cerevisae (EC38/CIS2/YLR299w; Mehdi et al., 2001) was used to search for homologs in the C. graminicola genome (Broad Institute, Cambridge, MA). Three putative GGTs and their loci tags were CgGGT1 (GLRG_09590), CgGGT2 (GLGR_05260) and CgGGT3 (GLRG_04161). CgGGT1 was cloned because qRT-PCR analysis indicated that it was the most highly expressed GGT gene over a range of conditions. Based on the genome sequence, a 4172 bp fragment, including the flanking 1240 bp upstream sequence, the predicted full-length

![Fig. 1. Construction of a CgGGT1 gene replacement vector and confirmation of C. graminicola GGT strains.](image-url)

(A) The pGGT1A knockout vector was constructed by PCR amplification of CgGGT1 from genomic DNA. The ∼1.5 kb upstream and downstream flanking sequences were amplified using GGT1 KO 5′ and GGT1 KO 3′ primer pairs, respectively, and cloned into pCB1636 at the border cloning sites. The intron in the genome (Broad Institute, Cambridge, MA). Three putative GGTs and their loci tags were CgGGT1 (GLRG_09590), CgGGT2 (GLGR_05260) and CgGGT3 (GLRG_04161). CgGGT1 was cloned because qRT-PCR analysis indicated that it was the most highly expressed GGT gene over a range of conditions. Based on the genome sequence, a 4172 bp fragment, including the flanking 1240 bp upstream sequence, the predicted full-length
2.2.6. Complementation of CgGGT1 restriction analysis, Southern blot and sequencing. The internal mants were single-spore purified, and then confirmed by PCR with primers (GGT1 seq 1 through 8, in Table 1) as indicated in Table 1. PCR was performed as in Section 2.2.2 except for the 4.2 kb fragment in pGEM-GGT1 (Section 2.2.4). The pGEM-GGT plasmid was digested with restriction sites added for cloning are bolded and underlined. The resulting PCR product was cloned into the pGEM-T Easy vector (Promega) and named pGEM-GGT1 4.2 kb (Table 1). PCR was performed with GGT1 seq5 primers, which amplify the 3′-glutamyl-γ-glutamyltransferase activity was quantified with pGNT1 sequence was confirmed. This vector was transformed into CgGGT1 Δ and wild type strains, for complementation and over-expression of CgGGT1, respectively, as described in Section 2.2.5 except that the transformation mixture was added to a minimal medium containing 17% (w/v) sucrose, 0.017% yeast nitrogen base without amino acids and ammonium sulfate, and 2% agar. After 24 h, a top agar with 2% glucose instead of sucrose and 200 μg/ml glufosinate ammonium (Sigma) was added. Putative glufosinate-resistant transformants were single-spore purified and transformants were further examined by PCR with the bar primers and restriction enzyme digestion analysis.

Table 1

<table>
<thead>
<tr>
<th>Name of primer pair</th>
<th>Sequence (5′→3′)</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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</thead>
<tbody>
<tr>
<td>Cloning and sequencing of CgGGT1</td>
<td>pGGT1 4.2 kb</td>
<td>AGCCCTTGAGGTTTTACCATG</td>
<td>AAGGGGCGCAGTCAGGAC</td>
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<tr>
<td>pUC M13/GGT1 seq1</td>
<td>CCGCAGGTTTTTCCCCAGGAA</td>
<td>TTTTCCGAGACTCCACCAAC</td>
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</tr>
<tr>
<td>GGT1 seq2</td>
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<td>ACAGTCGAGCTCGAGGCA</td>
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</tr>
<tr>
<td>GGT1 seq3</td>
<td>CGCCGCCCTCTAGAAAGT</td>
<td>GGCGGGAACGGCTAGCTA</td>
<td></td>
</tr>
<tr>
<td>GGT1 seq4</td>
<td>GTGCCGAGCTCTGCTGTTGA</td>
<td>ATGCTGCTGCTGCTGCT</td>
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<tr>
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</tr>
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<td>GGT1 seq7</td>
<td>CGGCCACATTATATTCCGG</td>
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<tr>
<td>GGT1 seq8/pUC M13</td>
<td>CTGAGAACGTGCTTGA</td>
<td>TCACAGGACGAAACCTAGCT</td>
<td></td>
</tr>
</tbody>
</table>

a Restriction sites added for cloning are bolded and underlined. b Bar = GTCGACAGAAGATGATATTGAAGG GTCGACCTAAATCTCGGTGAC.

2.2.7. Southern blot hybridization analysis

The GGT1 probe (608 bp) was amplified from wild type DNA with GGT1 seq5 primers, which amplify the 3′ end of the ORF (1000–1608 bp from the start codon). A hygromycin-resistance fragment was amplified from pCB1534 with the Ghy B primers. DIG-labeled DNA was generated with the PCR DIG Probe Synthesis kit (Roche, Indianapolis, IN, USA). Genomic DNA (2 μg) that was digested with MscI and DIG-DNA molecular weight markers (Roche) were separated by gel electrophoresis and transferred onto a Hybond-N + membrane (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The probe was hybridized and detected using the DIG Chemiluminescent Detection kit (Roche) with an anti-DIG-AP antibody and CSPD as a substrate.

2.3. γ-glutamyltransferase (GGT) activity and protein determination

The γ-glutamyltransferase activity was quantified with γ-glutamyl-p-nitroanilide (GnPNA; Sigma) as the substrate molecule and glycylglycine (Sigma, St. Louis, MO, USA) as the donor molecule (Tate and Meister, 1985). In preliminary experiments, we confirmed that the assay was linear in the detection range. After grinding 30 mg of conidia or mycelia (Section 2.2.1), the disrupted tissue was suspended in 0.9 or 1.2 ml of TE buffer (100 mM Tris,
10 mM EDTA, pH 7.5 except when pH 8.5 indicated), respectively. Crude cell extracts were pipetted with tips with a wide 1.5-mm diam bore.

The GGT activity assay contained 100 μl of crude cell extract, 100 mM Tris at either pH 7.5 or pH 8.5, 10 mM EDTA, 20 mM glycyglycine, and either 2.5 mM GpNA or no GpNA (as a negative control for each treatment) in 750 μl. After samples were incubated on a rotator at 25 °C for 2 h for mycelia and 3 h for conidia, 50 μl of dichloromethane (DCM; Sigma) was added to the samples to precipitate lipids, and tubes were vortexed and centrifuged at 14,000 g for 5 min. The supernatant (550 μl) was transferred to a new tube containing 110 μl of glacial acetic acid to stop the reaction. The release of p-nitroaniline from GpNA was measured by absorbance at 410 nm (Genesys 10vis; Thermo Fisher Scientific, Madison, WI, USA); GpNA-free controls were used as blanks for each sample.

To normalize GGT activity mg⁻¹ protein h⁻¹, 50 μl of the same cell extracts in TE used for the GGT assay were diluted in 450 μl of TE and incubated at 37 °C for 30 min to solubilize protein. Duplicate 50 μl samples were then assayed for soluble protein by the BCA method with a bovine serum albumin standard (Thermo Scientific Pierce, Rockford, IL, USA). After incubation for 30 min at 37 °C, 20 μl of DCM was added, and the samples were vortexed and centrifuged before spectrophotometric assessment of the supernatant. There were four independent biological replicates for each treatment, with each replicate conducted as a separate trial.

2.4. Cultural phenotypes on media with either GSH or various compounds that cause oxidative stress

Vogel’s medium without nitrogen (www.fgsc.net/methods/vogelts.html) was obtained from the Fungal Genetics Stock Center. Compounds (Sigma) used as amendments were filter-sterilized. Hyphal plugs (5 mm diam) from the margin of a 7-day-old colony on PDA were used to inoculate media.

To determine if CgGGT1 can utilize GSH as a N source, Vogel’s agar without ammonium nitrate (which contains trace quantities of ammonium from ferrous ammonium sulfate) (Vogel’s-N) was supplemented with 10 mM 2-(N-morpholino) ethanesulfonic acid (MES, pH 6.1) and either 0, 3 or 6 mM GSH. Petri dishes (55 mm diam) containing 10 ml Vogel’s-N agar were inoculated with the CgGGT1 strains and incubated in the dark at 28 °C for 4 days. To quantify mycelial biomass, Petri plates (100 mm diam) containing 12 ml of Vogel’s broth (which contains 25 mM ammonium nitrate) or Vogel’s broth-N with 10 mM MES (pH 6.1) and either 3 or 6 mM GSH were inoculated with two hyphal plugs per dish and incubated as above for 3 and 4 days, respectively. After incubation, agar plugs were removed and mycelial mats were collected onto pre-weighed Miracloth under vacuum, rinsed with water, and lyophilized. There were four independent biological replicates per treatment. Each replicate consisted of two or three dishes for treatments containing Vogel’s broth without and with GSH, respectively. Treatments were incubated and processed in a randomized complete block design; the block had no significant effect. Two independent trials were conducted.

To determine if CgGGT1 is involved in protection from oxidative stress, molten PDA was either unamended or amended with the following range of concentrations of compounds: 0.1–2 mM tert-butyl hydroperoxide; 30–300 μM menadione sodium bisulfite, 5–100 ppm chlorothalonil; 5–100 ppm mancozeb; and 5–40 mM cadmium chloride. After Petri dishes (55 mm diam) containing 10 ml of agar were inoculated with a hyphal plug, dishes were incubated in the dark at 28 °C. Hyphal extension and colony appearance was evaluated daily.

Agar media containing 5% potato dextrose broth also was unamended or amended with the following range of concentrations of compounds: 10–25 μM toluidine blue, 4–8 μM rose bengal, and 25–100 μg/ml Calcofluor white. Plates were inoculated with approximately 1 × 10⁴ washed conidia in 50 μl droplets. One set of plates containing toluidine blue and rose bengal were incubated in the dark at 28 °C for 8 h; another set of the same plates were incubated 4 h under fluorescent light, and then incubated for another 4 h in the dark. Plates with Calcofluor white were incubated at 28 °C for 8 h under fluorescent light. After incubation conidia were stained with lacto-phenol cotton blue, and the percentage germination was assessed.

2.5. Virulence assay

A highly anthracnose-resistant inbred (H99) corn, a highly susceptible inbred (Mo940), and a moderately susceptible hybrid (Yellow Jubilee) were used. H99 and Mo940 were obtained from L. Vaillancourt (Dept. Plant Pathology, University of Kentucky) and Yellow Jubilee was purchased from Livingston Seed Co. (Columbus, Ohio, USA). Plants were grown in 4-L plastic pots with UC Davis soil mix (Drewitz and DiTomaso, 2004), fertilized weekly and maintained in a greenhouse at 25–28 °C. Seven-week-old plants at the V10 or V11 vegetative stage (Ritchie et al., 1993) were inoculated. After the leaves from the first and second internode were removed, the sheath of the 3rd leaf was surface-disinfected (Clorox Disinfecting Wipe, Clorox, Oakland, CA, USA), rinsed with sterile water and blotted dry. The sheath was wounded with a sterile 4-mm diam cork borer. To inoculate a 6-mm diam plug of mycelium (without the underlying agar) was removed from the growing edge of a 1-week-old colony on PDA, placed on the sheath 9–11 cm from the internode and midway between the major vein and the edge of the sheath, and wrapped with Parafilm (Pechinery Plastic, Menasha, WI, USA). After 10 days the inoculated sheath was removed, taped onto blue paper, and scanned with an HP Scanjet 5530 Photosmart Scanner (Palo Alto, CA, USA) at 600 dpi. JPEG files were digitally analyzed based on hue for the percentage of symptomatic tissue with Assess 2.0 Image Analysis Software (American Phytopathological Society Press, St. Paul, MN, USA) and manually checked for accurate classification of tissue. There were either ten or 15 replicate plants per isolate per trial.

2.6. Quantification of reduced glutathione (GSH) and oxidized glutathione (GSSG) with reverse-phase HPLC with electrochemical detection

We followed methods described in Bello et al. (2012) except that the mobile phase contained 25 mM sodium phosphate, 50 μM octane sulfonic acid, and 0.75% acetonitrile adjusted to pH 2.7 with phosphoric acid. The quantity of GSH and GSSG was normalized to soluble protein (Bello et al., 2012). There were three independent biological replicates per treatment, with each replicate conducted as a separate trial.

2.7. Statistical analysis

Data were analyzed as appropriate by one- or two-way ANOVA (JMP8, SAS Institute, Cary, NC, USA). Homogeneity of variance was examined using Levene’s test. When the data failed Levene’s test (P < 0.05), log₁₀-transformed data were used to satisfy the assumption of homoscedasticity for ANOVA. Means separation was performed using Tukey’s HSD (α = 0.05). Contrast analysis was used for specified comparisons. Means ± SE are shown in the text, except when indicated.
3. Results

3.1. Identification of C. graminicola GGT orthologs and confirmation of in silico gene structure of CgGGT1

Three putative GGT genes were identified in the C. graminicola genome. Compared to the predicted 660 aa sequence of GGT in S. cerevisiae (CIS2), CgGGT1 was 33% identical over 530 aa, CgGGT2 was 34% identical over 528 aa, and CgGGT3 was 26% identical, but only over 157 aa. All three CgGGTs have a GGT domain (NCBI CDD: Pfam PF01019; Marchler-Bauer et al., 2011) with the following conserved sequence in the catalytic region: T(STA)HX(ST) (prosite.expasy.org/PDOC00404). The CgGGT1 (1777 bp, GenBank JQ413976) and CgGGT2 (2122 bp) genes are located on separate arms of chromosome 2, and CgGGT3 (2111 bp) gene is located on chromosome 7. The in silico sequence of CgGGT1 predicted a single intron. Using genomic and cDNA from C. graminicola conidia, we confirmed that CgGGT1 has a 1777 bp ORF with an 84 bp intron (GenBank JQ413977). CgGGT2 has six predicted introns; the last intron was confirmed using RT-PCR with primers that span the predicted exon–exon junction. Using the consensus sequence TGAN 7N (Springael and Penninckx, 2003) and the algorithm at The Saccharomyces cerevisiae Promoter Database (http://rulai.cshl.edu/SCP), potential GATA regulatory elements were identified in all three CgGGTs: CgGGT1 (5 motifs), CgGGT2 (10), and CgGGT3 (7).

3.2. Expression of CgGGTs in the wild type C. graminicola in media and in planta

All three CgGGTs were transcribed in wild type conidia (data not shown). All strains were tested at two stages (data not shown). In the wild type, nitrogen-starved conidia had significantly (\( \alpha = 0.05 \)) more (4.5X) CgGGT1 transcripts than those in nitrogen-rich media. Based on comparisons of the 95% confidence intervals (CI95), the transcription of CgGGT1 in the wild type was significantly greater than CgGGT3 during nitrogen starvation.

To quantify expression of CgGGTs during plant infection, quantitative RT-PCR (Fig. 4) also was used to analyze infected tissue in the biotrophic phase 1 day post inoculation (dpi) when host tissue was asymptomatic, and in the necrotrophic phase (4 days post inoculation (dpi)) when host tissue was necrotic. All three CgGGT transcripts were detected at both stages; there were no significant differences in levels of any of the three GGTs in the biotrophic versus the necrotrophic phase (\( P_{CgGGT1} = 0.72; P_{CgGGT2} = 0.77; P_{CgGGT3} = 0.18 \)). Because CgGGT1 expression was greater in media and in planta, it was chosen for further characterization.

3.3. Generation and selection of CgGGT1 knockout, complemented knockouts and an over-expressing strain

To determine the function of CgGGT1 in C. graminicola, two deletion strains (Fig. 1A, named CgGGT1Δ-54 and CgGGT1Δ-83), complemented deletion strains, and an over-expressing (CgGGT1OE) transformant were created. Southern blot hybridization analysis confirmed that the two deletion strains had a homologous replacement of CgGGT1 with the hph gene (Fig. 1B). Furthermore, RT-PCR analysis demonstrated that neither mutant produced CgGGT1 transcripts, in contrast to the wild type (Fig. 1C). These data were confirmed with qRT-PCR (Fig. 2 upper panel, and Fig. 3A).

In 12-h-old germlings grown in both Fries nitrogen-rich and Fries nitrogen-deficient media, the two CgGGT1Δ strains had no CgGGT1 expression (Fig. 2). The two complemented CgGGT1Δ strains had wild-type levels of CgGGT1 transcription. The CgGGT1OE strain had significantly (\( \alpha = 0.05 \)) more CgGGT1 transcripts than any of the other strains. Similarly, in 5-day-old mycelia from Fries nitrogen-rich medium, no CgGGT1 expression was detected in the two CgGGT1Δ strains (Fig. 3A). The two complemented CgGGT1Δ strains and the CgGGT1OE strain had significantly (\( \alpha = 0.05 \)) more CgGGT1 expression than any of the other strains, including the wild type. This finding indicates that integration of an intact copy of CgGGT1 restores CgGGT1 activity in the knockout mutants, and that introduction of additional CgGGT1 into the wild type genome results in higher expression levels of CgGGT1 mRNA. Significant (\( \alpha = 0.05 \)) increases and decreases in CgGGT1 expression had no effect on mRNA transcript levels for either CgGGT2 or CgGGT3 in germlings (Fig. 2) or mycelia (Fig. 3B and C).

3.4. Production of GGT in conidia and mycelia by the CgGGT1

GGT activity was detected in mycelia (Fig. 3D) and conidia (Fig. 5A) in all strains: the wild type, CgGGT1OE, two CgGGT1Δ, and the two complemented CgGGT1Δ strains. However, based on...
Fig. 3. mRNA expression of the three C. graminicola γ-glutamyltransferases (GGTs), GGT activity and glutathione (GSH) concentration in C. graminicola mycelia. Five-day-old mycelia from the wild type, the CgGGT1A mutants, the complemented CgGGT1A mutants and CgGGT1-over-expressing strain were collected from cultures grown in Fries medium. Expression analysis of (A) CgGGT1, (B) CgGGT2, and (C) CgGGT3 mRNA was determined by quantitative RT-PCR. Expression of each CgGGT was normalized to β-tubulin and relative changes in expression were calculated by the 2^{-ΔΔCt} method. mRNA expression values were log_{10}-transformed to achieve homoscedasticity, and are presented on a log scale. Means ± SE of four biological replicates are shown. (D) GGT activity at pH 7.5 was measured spectrophotometrically using γ-glutamyl-p-nitroanilide as donor and glycylglycine as acceptor substrate. GGT activity values were log_{10}-transformed to achieve homoscedasticity. De-transformed means of four biological replicates and error bars with the 95% confidence intervals are shown. (E) GSH content was measured by HPLC with electrochemical detection. Means ± SE of three biological replicates are shown. For panels A and D, which had P < 0.05, bars with the same letter are not significantly different by Tukey’s HSD, α = 0.05.

Fig. 4. mRNA expression of the three C. graminicola γ-glutamyltransferases (GGTs) in planta. Sheath leaves of seven-week-old highly susceptible corn cv. Mo940 were inoculated with a mycelial agar plug. Lesions were excised from sheaths 4 and 6 days post-inoculation (dpi). Expression analysis of CgGGT1, CgGGT2, and CgGGT3 was determined by quantitative RT-PCR. Expression of each CgGGT was normalized to β-tubulin and relative changes in expression were calculated by the 2^{-ΔΔCt} method. Data were log_{10}-transformed and results are presented on a log scale. Means ± SE of four biological replicates are shown.

CgGGT1OE, mycelial homogenates were a significantly richer source of GGT than conidial homogenates (compare Figs. 3D and 5A). For example, the GGT CgGGT1 OE in the wild type strain was 18–41 and 175–230 nmol p-nitroanilide mg^{-1} protein h^{-1}, for the conidia and mycelia, respectively. Mycelia of the two CgGGT1A strains had significantly less GGT activity (α = 0.05), and only 32% ± 2% (n = 8) of the wild type (Fig. 3D). Similarly, the conidia of the two CgGGT1A strains had significantly less GGT activity (α = 0.05), and only 14% ± 1% (n = 8) of the wild type strain (Fig. 5A). Consequently, we estimate that CgGGT1 produces 68% ± 2%, and 86% ± 1% of the total GGT activity in mycelia and conidia, respectively.

We compared the estimates of the GGT activity in the conidia of all strains assayed at pH 7.5 and 8.5. There was no significant strain x pH interaction (P = 0.97), no significant pH effect (P = 0.47), and a highly significantly strain effect (P = 0.0001). Across all strains, mean nmol p-nitroanilide mg^{-1} protein h^{-1} was 52 ± 5 and 46 ± 8 at pH 7.5 and 8.5, respectively.

GGT activity in mycelia was significantly increased (α = 0.05) 163% ± 16% (n = 8) in the complemented CgGGT1A strains and 314% ± 25% in the CgGGT1OE (n = 4) strain compared with the wild type (Fig. 3D). An examination of the levels of CgGGT1 transcription and the level of GGT activity in the six strains indicated a significant (P = 0.004) correlation (r = 0.95) between abundance of CgGGT1 transcript and enzymatic activity. Similarly, in the six strains there was a significant (P = 0.002) correlation (r = 0.996) of GGT activity in mycelia and conidia. For example, GGT activity in conidia was significantly increased (α = 0.05) 205% ± 22% (n = 8) in the complemented strains and 838% ± 205% (n = 4) in the CgGGT1OE strain compared with the wild type. Consequently, the strains provided a range of mean GGT activity from 4 to 209 nmol p-nitroanilide h^{-1} mg^{-1} protein in conidia, and from 58 to 623 nmol p-nitroanilide mg^{-1} protein h^{-1} in the mycelia.

3.5. Effect of nitrogen starvation on the expression of CgGGTs mRNA

A two-way ANOVA of transcript levels of CgGGTs in germlings of the six strains in nitrogen-rich versus nitrogen-deficient media
GGT activity values were log10-transformed to achieve homoscedasticity. A significant expression of \( \text{P} \) strains, nitrogen starvation significantly \( \text{CgGGT1} \) in \( \text{C. graminicola} \) nitrogen deficiency, the transcription in germlings \( \text{CgGGT1} \) apparently enables utilization of GSH as a nitrogen source (Supplemental Fig. 1). Although nitro-
deficient \( \text{Vogel's agar medium amended with either 0, 3 or 6 mM GSH as the nitrogen source} \) and over-expression strains had significantly \( \text{CgGGT1} \) over-expressing strains had approximately one-quarter and 3 or 6 mM GSH, both \( \text{CgGGT1A} \) strains had significantly less biomass \( (P < 0.0001) \), and only 23% ± 2% and 41% ± 3% \( (\text{Cl}_{95}, n = 8) \) of the growth of the wild type, respectively. The \( \text{CgGGT1OE} \) produced significantly \( (P < 0.0001) \) more biomass than any of the other strains in 3 and 6 mM GSH, and had 272% ± 16% and 281% ± 16% \( (\text{Cl}_{95}, n = 4) \) more biomass, respectively, compared with the wild type. These results suggest that \( \text{CgGGT1} \) is involved in the utilization of GSH as a nitrogen source during nitrogen deficiency. In contrast, in a nitrogen-rich medium with 25 mM ammonium nitrate, the strains grew similarly except that one of the complemented strains produced significantly \( (\alpha = 0.05) \) less biomass than the wild type.

3.7. \( \text{CgGGT1} \) is not required for virulence of \( \text{C. graminicola in a wounded leaf assay} \)

To determine whether \( \text{CgGGT1} \) is a virulence or pathogenicity factor, corn plants in a greenhouse were inoculated with the wild type and the set of mutant strains. Because \( \text{C. graminicola} \) is a hemi-biotroph, two inoculation assays were devised in which an agar plug with mycelia was placed on either intact (to allow for initial biotrophic growth) or wounded sheath leaves of anthracnose-susceptible corn. In the unwounded treatments \( \text{data not shown} \), the variance in the percentage of symptomatic sheath leaves in all treatments was too high to make inferences. Consequently, only the wounded-sheath assay was used for subsequent experiments.

In these experiments, the anthracnose-resistant corn cv. H99 inoculated with the wild type \( \text{and the other strains} \) did not develop symptoms by 10 dpi \( \text{data not shown} \), whereas the highly susceptible cv. Mo940 had approximately four times more symptomatic tissue than the moderately susceptible Yellow Jubilee \( \text{Table 3}. \) In the susceptible hosts, symptoms of the disease appeared within 5 dpi. Together these results suggest that the wounded-sheath assay reflects disease in the field. All mutant strains were as virulent as the wild type on both cv. Mo940 and Yellow Jubilee \( (\alpha = 0.05; \text{Table 3}) \). Thus, \( \text{CgGGT1} \) is not required for virulence of \( \text{C. graminicola in wounded corn sheaths} \).

3.8. Deletion and over-expression of \( \text{CgGGT1} \) has no effect on glutathione levels and \( \text{CgGGT1A in vitro growth assays} \)

To determine whether GSH can be utilized for growth during nitrogen deficiency, the \( \text{CgGGT1} \) strains were grown in nitrogen-deficient Vogel's agar medium amended with either 0, 3 or 6 mM GSH as the nitrogen source \( \text{Supplemental Fig. 1}. \) Although nitrogen-deficient, the medium has trace quantities of nitrogen from both contaminants in the agar and from ammonium in ferrous ammonium sulfate. Consequently, there is some growth at 0 mM GSH and it is indistinguishable for all strains \( \text{Supplemental Fig. 1}. \); this supports the hypothesis that the transformants are not pleiotropically affected. When the medium was amended with either 3 or 6 mM GSH, the \( \text{CgGGT1A} \) mutants grew less than the wild type, complemented \( \text{CgGGT1A} \) mutants, and the over-expressing strains. The over-expressing strain grew better than any of the other strains. On the agar plates, 6 mM GSH inhibited hyphal extension of all strains compared to 3 mM GSH. The experiment was modified in order to quantify biomass \( \text{Table 2}. \) When strains were grown in modified Vogel's broth amended with either concentrations of GSH in conidia and myce-
llings were indistinguishable with the \( \text{Cl}_{95} = 2.6–3.7 \) and 2.7–4.0 mmol GSH m\(^{-1}\) protein in wild type conidia and mycelia, respectively.

Based on either percentage conidial germination or in vitro growth assays, the \( \text{C. graminicola GGT1} \) strains did not have any differential sensitivity to oxidants \( \text{ertest-butyl hydroperoxide, menadi-
one, and toluidine blue and rose Bengal in the light} \), the heavy metal cadmium chloride, xenobiotics \( \text{chlorothalonil and manco-
zeb} \), and Calcofluor white \( \text{data not shown} \).
the first and second trial, respectively. Since the data from the two trials were similar, they were pooled for statistical analysis. Mean ± SE of 10 and 25 replicates from Mo940 and Yellow Jubilee, respectively, are shown. The assay with Yellow Jubilee was performed twice with 10 and 15 replicates for inoculated control plants were treated with an uncolonized plug of PDA. Ten days after inoculation, percentage of diseased area was determined using digital image analysis.

Table 2
Utilization of glutathione (GSH) as a nitrogen source by Colletotrichum graminicola wild type and strains affected in the γ-glutamyltransferase, CgGGT1.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Nitrogen source in Vogel’s modified broth</th>
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<tbody>
<tr>
<td></td>
<td>3 mM GSHb</td>
</tr>
<tr>
<td></td>
<td>6 mM GSHb</td>
</tr>
<tr>
<td></td>
<td>Standard 25 mM ammonium nitrate</td>
</tr>
<tr>
<td></td>
<td>Mycelia dry weight (mg ± SE) per dl brothc</td>
</tr>
<tr>
<td>Wild type</td>
<td>104 ± 4 c</td>
</tr>
<tr>
<td>CgGGT1::CgGGT1</td>
<td>97 ± 3 b</td>
</tr>
<tr>
<td>CgGGT1::CgGGT1Δ-54</td>
<td>255 ± 14 a</td>
</tr>
<tr>
<td>CgGGT1::CgGGT1Δ-83</td>
<td>243 ± 20 ab</td>
</tr>
<tr>
<td>CgGGT1 over-expressing</td>
<td>238 ± 6 ab</td>
</tr>
<tr>
<td></td>
<td>284 ± 7 a</td>
</tr>
<tr>
<td></td>
<td>293 ± 12 a</td>
</tr>
<tr>
<td></td>
<td>197 ± 10 ab</td>
</tr>
</tbody>
</table>

a Petri dishes containing the indicated broth were inoculated with two mycelial plugs of the indicated strain, and incubated in the dark for either 3 (25 mM ammonium nitrate) or 4 days (+GSH). Data shown are from two experiments each with four replicates. Tukey’s mean separations were the same when results from each trial were analyzed separately.

b Media were buffered with 10 mM MES buffer, pH 6.1.

c Within a column, means followed by the same letter were not significantly different (α = 0.05, Tukey’s test).

Table 3
Virulence of Colletotrichum graminicola wild type and strains affected in the γ-glutamyltransferase, CgGGT1.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Cultivar</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Highly susceptible cv. Mo940</td>
</tr>
<tr>
<td></td>
<td>Moderately susceptible cv. Yellow Jubilee</td>
</tr>
<tr>
<td></td>
<td>Symptomatic area (% ± SE)b</td>
</tr>
<tr>
<td>Wild type</td>
<td>10.1 ± 0.6 a</td>
</tr>
<tr>
<td>CgGGT1::CgGGT1</td>
<td>9.5 ± 0.8 a</td>
</tr>
<tr>
<td>CgGGT1::CgGGT1Δ-54</td>
<td>11.6 ± 0.6 a</td>
</tr>
<tr>
<td>CgGGT1::CgGGT1Δ-83</td>
<td>9.0 ± 0.7 a</td>
</tr>
<tr>
<td>CgGGT1 over-expressing</td>
<td>11.2 ± 0.5 a</td>
</tr>
<tr>
<td>Mock-inoculated control</td>
<td>0.2 ± 0.2 b</td>
</tr>
</tbody>
</table>

a Seven-week-old sheath leaves of corn were inoculated with a mycelial plug from a 1-week-old colony on potato dextrose agar (PDA) of the indicated strain. Mock-inoculated control plants were treated with an uncolonized plug of PDA. Ten days after inoculation, percentage of diseased area was determined using digital image analysis. Mean ± SE of 10 and 25 replicates from Mo940 and Yellow Jubilee, respectively, are shown. The assay with Yellow Jubilee was performed twice with 10 and 15 replicates for the first and second trial, respectively. Since the data from the two trials were similar, they were pooled for statistical analysis.

b Within a column, means followed by the same letter were not significantly different (α = 0.05, Tukey’s test).

inhibits hyphal extension in all strains in comparison to 3 mM GSH, and that GGT helps to ameliorate the toxicity at 6 mM GSH, presumably by GSH catabolism. However, in the quantified biomass produced in broth (Table 2) there was no evidence that 6 mM GSH inhibited growth since biomass produced in 6 mM GSH was not significantly (α = 0.05) less than biomass produced in 3 mM GSH. Regardless, the experiments support that CgGGT1 is involved in utilization of GSH as a N source in a N-deficient medium, and is not simply reducing any GSH toxicity. In the Supplemental Fig. 1, growth of all strains except the two knockouts is greater in 3 mM GSH than in 0 mM GSH. Jaspers et al. (1985) demonstrated that S. cerevisiae utilized radioactively-labeled GSH as a nitrogen source when grown with the poor nitrogen sources urea and glutamate. Indirect evidence including GGT transcript expression and GGT enzymatic activity supports the view that S. cerevisiae GGT (Cis2p) (Mehdi and Penninckx, 1997; Springael and Penninckx, 2003) and S. pombe Ggt1 and Ggt2 (Kang et al., 2005; Kim et al., 2005; Song and Lim, 2008) allows utilization of GSH as a nitrogen source during nitrogen deficiency. In S. cerevisiae and S. pombe, GSH is imported via the high-affinity GSH transporters HGT1 and Pgt1, respectively, in the plasma membranes (Bourbouloux et al., 2000; Thakur et al., 2008); these transporters are found only in plants and some fungi (Bourbouloux et al., 2000). S. cerevisiae HGT1Δ strains were unable to take up radioactively-labeled GSH from the medium, and HGT1Δ GSH1Δ strains were not viable (Bourbouloux et al., 2000). An in silico search indicates that C. graminicola has 13 potential homologs to HGT1 with E = 0.0.

Similar to the Sodariomycete C. graminicola, Sclerotinia sclerotiorum, a necrotrophic Leotiomyecete in the Pezizomycota, has three GGTs. A phylogenetic analysis indicates that SsGgt1 (gene SS1G_14127.3), SsGgt2 (gene SS1G_10940.3), and SsGgt3 (gene SS1G_05330.3) are homologs of CgGGT1, CgGGT2 and CgGGT3, respectively (Bello and Epstein, 2013). Recently, Li et al. (2012) demonstrated that knockouts of S. sclerotiorum SsGgt1 had reduced virulence in unwounded tomato leaves, but were as virulent as the wild type in wounded leaves. We had similar results in wounded assays of the CgGGT1 mutant strains in corn sheath leaves, but were unable to quantify virulence in unwounded assays. Consequently, it is possible that wounding releases sufficient nitrogen to satisfy the pathogen growth demands, and that in unwounded plants, GSH might be a nitrogen source, particularly during the biotrophic phase of pathogenesis when nitrogen is limited (Brown et al., 2008; Pellier et al., 2003). In corn seedling leaves, GSH concentrations were estimated between 0.35 and 1 mM (Lay and Niland, 1985).

In germlings, all three CgGGT genes were significantly (α = 0.05) induced by nitrogen-starvation. Although CgGGT1 had the highest expression in both nitrogen-rich and -deficient conditions, expression of CgGGT3 was the most dependent on nitrogen deficiency with a ~12-fold increase; CgGGT1 and CgGGT2 were up-regulated ~4.5-fold in nitrogen-deficient conditions. GGT transcription in S. cerevisiae and S. pombe is affected by nitrogen and carbon starvation (Kang et al., 2005; Kim et al., 2005; Springael and Penninckx, 2003). In S. cerevisiae, Cis2 is up-regulated ~9-fold in cells grown in a nitrogen-poor source (e.g., glutamate) compared with those grown in a good nitrogen source such as ammonium sulfate (Kumar et al., 2003b). Interestingly, although the wild type CgGGT1 was up-regulated in nitrogen-starved germlings, and the
C. graminicola wild type and CgGGT1Δ strains were transformed with a construct that contained the 5' and 3' flanking regions of CgGGT1, functional ectopic copies of CgGGT1 in the complemented and over-expressed strains were not up-regulated by nitrogen starvation.

GGT assays in cell homogenates indicate that CgGGT1 produces the most enzyme activity of the three GGTs in C. graminicola: approximately 86% ± 2% of the enzyme activity in conidia; 71% ± 2% of activity in 12-h-old germlings grown in Fries medium, a nitrogen rich medium; and 68% ± 1% of the enzyme activity in mycelia grown in Fries medium. In an analysis of the six strains, levels of CgGGT1 transcript levels are highly correlated with levels of enzyme activity. GGT activity was detectable in conidia, but was 7-fold higher in mycelia. In S. sclerotiorum, northern hybridization and mutational analysis indicated that SsGGT1 was primarily expressed in sclerotia and in infection cushions (Li et al., 2012), which are not produced by C. graminicola.

Expression of CgGGT2 and CgGGT3 is independent of expression of CgGGT1. The quantitative RT-PCR data shows that deletion of CgGGT1 abolished expression of CgGGT1 mRNA expression, but had no effect on the transcript levels of CgGGT2 and CgGGT3. Similarly, over-expression of CgGGT1 had no effect on CgGGT2 and CgGGT3. In S. cerevisiae, only ~10% of duplicated genes were up-regulated when their paralogs were deleted, and only under certain conditions where the gene’s function was required (DeLuna et al., 2008).

Estimates of C. graminicola GGT activity in conidia were not significantly different (P = 0.47) when assayed at pHs 8.5 and 7.5. With S. cerevisiae extracts, the GGT-catalyzed release of p-nitroanilide from γ-glutamyl-p-nitroanilide occurred via hydrolysis with an optimum at pH 7.0 and via transpeptidation with an optimum of pH 8.5–9.5 (Penninckx et al., 1980; Penninckx and Jaspers, 1985). The fact that estimates of GGT activity at pH 8.5 were on average 88% of those at pH 7.5 supports the hypothesis that the pH 7.5 assay provides a reasonable assessment of GGT activity (Ubiyovk et al., 2006), and one that is closer to physiological pH.

Fig. 6. Chromatogram of reverse phase HPLC separation of 10% perchloric acid-soluble extracts with electrochemical detection. Chromatogram (A) of low-molecular weight thiol standards: 25 pmol of each of cysteine (cys; 3.9 min); cysteinylglycine (cys-gly; 7.8 min); reduced glutathione (GSH; 8.9 min); and oxidized glutathione (GSSG; 32.2 min). (B) Extracts of C. graminicola conidia and (C) mycelia representing the equivalent of approximately 36 µg dry weight tissue. (D) Chromatogram of half of the conidial sample in B co-injected with 12.5 pmol GSH and GSSG standards. Only GSH was detected in conidial and mycelial lysates.
yeast GGT homologs and C. graminicola CgGGT1 could be due to either differing function between the GGT clades or to GGT expansion in the Pyrenomycetes particularly in CgGGT2 (Bello and Epstein, 2013), or differences in assay conditions. In S. cerevisiae, one third of the duplicated genes have reduced expression and some functional redundancy (Qian et al., 2010). In addition, Springer et al. (2010) suggest that the majority of S. cerevisiae proteins are expressed at higher levels than necessary. Consequently, we predict that double- and triple-CgGGT knockouts may show defective phenotypes in planta and additional phenotypes in vitro. Such mutants would allow further insights into the role of C. graminicola GGTs in pathogenicity, during nitrogen or sulfur starvation, and in catabolism of GSH.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fgb.2012.11.007.

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