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

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ABSTRACT

Gamma-glutamyltransferase (GGT, EC 2.3.2.2) cleaves the γ-glutamyl linkage in glutathione (GSH). Three GGTs in the hemibiotrophic plant pathogen *Colletotrichum graminicola* were identified *in silico*. GGT mRNA expression was monitored by quantitative reverse-transcriptase PCR. Expression of all three genes was detected *in planta* during the biotrophic and necrotrophic stages of infection. Of the three GGTs, *CgGGT1* mRNA (from gene GLRG_09590) was the most highly expressed. All three GGT mRNAs were up-regulated in wild type nitrogen-starved germlings in comparison to non-starved germlings. *CgGGT1* was insertionally mutagenized in *C. graminicola*, complemented with the wild type form of the gene, and over-expressed. Enzyme assays of two independent *CgGGT1* knockouts and the wild type indicated that CgGGT1 is the major GGT and accounts for 86% and 68% of total GGT activity in conidia and mycelia, respectively. The over-expressing strain had 8-fold and 3-fold more enzyme activity in conidia and mycelia, respectively, than the wild type. In an analysis of the GGT knockout, complemented and over-expressing strains, *GGT1* transcript levels are highly correlated ($r = 0.95$) with levels of total GGT enzyme activity. *CgGGT1* and *CgGGT2* genes in strains that had ectopic copies of *CgGGT1* were not up-regulated by nitrogen-starvation, in contrast to the wild type. Deletion or over-expression of *CgGGT1* had no effect on mRNA expression of *CgGGT2* and *CgGGT3*. In broth in which 3 and 6 mM glutathione (GSH) was the nitrogen source, the *CgGGT1* over-expressing strain produced significantly ($P < 0.0001$) more biomass than the wild type and complemented strains, whereas the *CgGGT1Δ* strains produced significantly ($P < 0.0001$) less biomass than the wild type strain. This suggests that CgGGT1 is involved in utilizing GSH as a nitrogen source. However, deletion and over-expression of *CgGGT1* had no effect on either virulence in wounded corn leaf sheaths or GSH levels in conidia and mycelia. Thus, the regulation of GSH concentration is apparently independent of CgGGT1 activity.

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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 (Penninckx 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 (Penninckx 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 Penninckx, 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 (Penninckx 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*

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Ggt2 is localized in the endoplasmic reticulum (Jaspers and Penninckx, 1984; Matsuyama et al., 2006). GSH is reportedly present in 5–10 mM concentrations in vacuoles of *S. cerevisiae* (Jaspers and Penninckx, 1984).

GGTs in *S. pombe* appear to be involved in the response to several types of oxidative stress. *ggt*2 transcripts are induced by H_2O_2, and *ggt*1 and *ggt*2 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_2O_2 (Springael and Penninckx, 2003); however, transcription of *CIS2* is not induced by 1 mM H_2O_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 GTT activity, was also more sensitive to 0.8 mM *tert*-butyl hydroperoxide than the wild type (Ubiyvovk et al., 2006). Park and associates (2004) showed that a *S. pombe* strain over-expressing endogenous *ggt1* grew in media containing 3 mM H_2O_2, in contrast to the wild type. However, a strain over-expressing *ggt2* had only slightly greater survival on media with 4 mM H_2O_2 compared with the wild type (Park et al., 2005). Gales et al. (2008) showed that a *CIS2*Δ mutant of *S. cerevisiae* tolerated more H_2O_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_2O_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; Mehdi and Penninckx, 1997) and in *S. pombe* (Song and Lim, 2008). GSH levels and GGT activity, increased during nitrogen starvation in *S. cerevisiae* (Mehdi 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 (Mehdi 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, lactose, 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 *CgGGT*s. 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 (1.5×10^6) 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 scrapper 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 RNeasy 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 plug of mycelium (Section 2.5). After the indicated incubation, the mycelial plug and the underlying sheath tissue were discarded. The sheath tissue adjacent to the plug was harvested using a 12-mm diam cork borer. 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 $MgCl_2$, 200 μM each of the dNTP's, 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 (βtub2 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 $2^{-\Delta Ct}$ 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 (germlings 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* (*ECM38/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 locus tags were *CgGGT1* (GLRG_09590), *CgGGT2* (GLGR_05260) and *CgGGT3* (GLGR_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

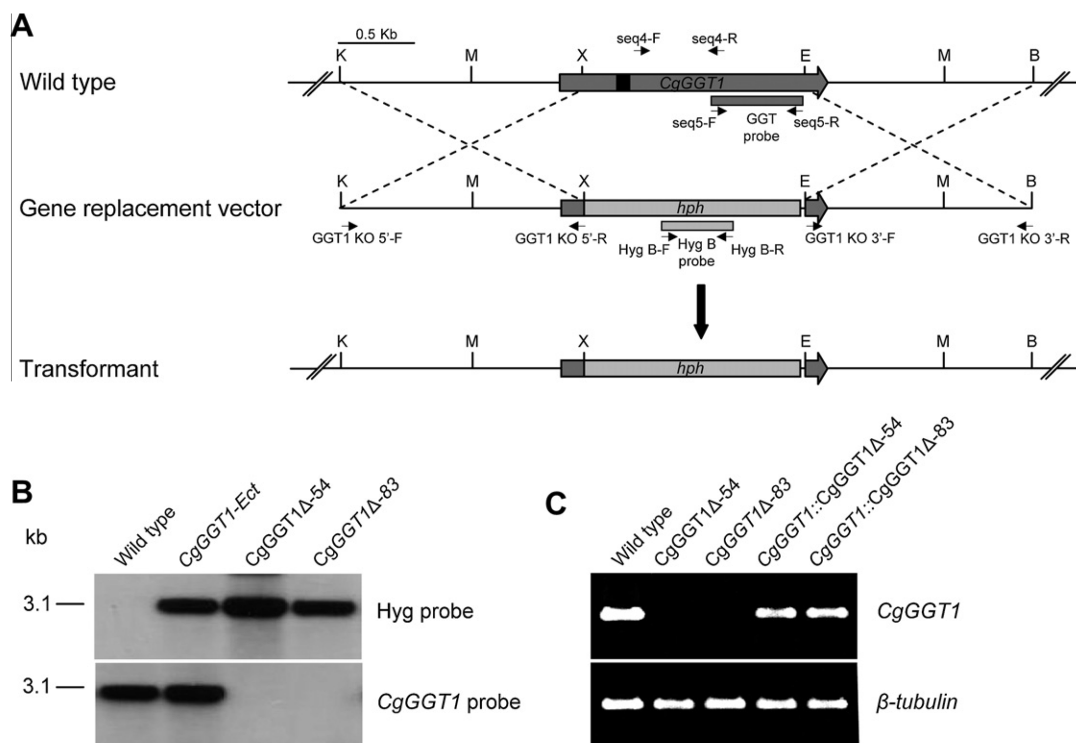

Fig. 1. Construction of a *CgGGT1* gene replacement vector and confirmation of *C. graminicola GGT* strains. (A) The pGGT1Δ knockout vector was constructed by PCR amplification of *CgGGT1* from genomic DNA. The ~1.5-kb upstream and downstream flanking sequences were amplified using GGT1 KO 5′ and GGT1 KO 3′ primer pairs, respectively, and cloned into pCB1636 at the *hph* border cloning sites. The intron in *CgGGT1* is represented by the black box. Primers are indicated by arrows. The restriction enzymes are abbreviated as K (*Kpn*I), M (*Msc*I), X (*Xho*I), E (*Eco*RI), and B (*Bam*HI). (B) Southern hybridization analysis of the wild type M1.001 strain, ectopic transformant *CgGGT1-Ect*, and *CgGGT1*Δ mutants. Genomic DNA was digested with *Msc*I, separated electrophoretically, transferred to a membrane, and hybridized with a probe for *hph*, the hygromycin-resistance gene used for insertional mutagenesis. The same blot was stripped and hybridized with the *CgGGT1* probe. Digestion with *Msc*I generates a 3111 bp fragment in the wild type gene. (C) Reverse transcriptase (RT)-PCR analysis of *CgGGT1* expression of wild type, the *CgGGT1*Δ mutants, and the complemented *CgGGT1*Δ mutants. Total RNA was isolated from 7-day-old conidia. Oligo $(dT)_{20}$ primer was used for cDNA synthesis, and primers GGT1 seq4 were used for the subsequent PCR. The 602 bp RT-PCR product is absent in the *CgGGT1*Δ strains. β-tubulin (*TUB2*) primers (βtub2 RT) were used as an internal control; they produce an 829 bp amplicon that spans an exon–exon junction.

Table 1
PCR primers used in this study.

Name of primer pair	Sequence (5′ → 3′)[a]	
	Forward primer	Reverse primer
Cloning and sequencing of CgGGT1		
GGT1 4.2 kb	AGCCTTGGGTTTACCATCG	AAGGACGCCGTCAAGGAG
pUC M13/GGT1 seq1	CGCCAGGGTTTTCCCAGTCACGAC	TTTTCCCAGACTCGACAACC
GGT1 seq2	GGTCCTTGATGAGGCTCAG	ACACTGGACTCGGATGCGAC
GGT1 seq3	CCAGCCCGTCATGAAGCTCT	GGCGTGACGCTGTCCATGTA
GGT1 seq4[b]	CTGCACAAGAAGTACGGCAAGC	ATGTCCTTGGTGTACGCCGC
GGT1 seq5	ATCGACGAGGCCATGCGCTT	TCGAAAGCGGCCGTATTGGG
GGT1 seq6	CATCACCGCCACCATCCAGA	AAGACACCGCCACAGTCC
GGT1 seq7	CGGCCACTTATATTCGTGCG	GAAAGTCGTTTGGCCAGGT
GGT1 seq8/pUC M13	CTGGAACGTCGCGTTGAC	TCACACAGGAAACAGCTATGAC
GGT1 gene replacement, complementation and over-expression vectors		
GGT1 KO 5′	TTTT**GGTACC**TGGGTTTTGAAACTACGGTAGCCC	TTTT**CTCGAG**GAGGTCGGTGCCAATGCGG
GGT1 KO 3′	TTTT**GAATTC**CGACCAGCTACCTCCAGACGCTGG	TTTT**GGATCC**TCTCGGGCCTCCTACGCACG
Hyg B	GTCCTGCGGGTAAATAGCTG	ACATTGTTGGAGCCGAAATC
bar	GTCGACAGAAGATGATATTGAAGG	GTCGACCTAAATCTCGGTGAC
"Conventional" reverse transcriptase (RT)-PCR[b]		
βtub2 RT	ATGAGCGTCTACTTCAACGAAGC	TGGCGACCTTACCACGGAA
Quantitative RT-PCR		
qβtub2–5	GTCGTTCCCTCCCCTAAGGTTT	GCTTAAGAGTACGCATGCAGATGTC
qβtub2–6	ACGGCGACCTGAACCATCTC	CGAATCCGACCATGAAGAAGTG
qGGT1–5	CCCAGAACACGTCGTGGTACA	ATGAGCTGCGAGCCGAAGA
qGGT2–2	TACGCCAGCGAAGGCTTC	GTCGTTCATCTCGTTGTTGAGG
qGGT3–7	CGCGCCAAGCTCTTCGA	CCGCCGTAGTTGCTGTTGA

[a] Restriction sites added for cloning are bolded and underlined.
[b] GGT1 seq4 primers were used for both sequencing and for "Conventional" RT-PCR.

1777 bp *CgGGT1* ORF, and the 1155 bp downstream flanking sequence, was amplified by PCR from genomic DNA with primers GGT1 4.2 kb (Table 1). PCR was performed as in Section 2.2.2 except 0.02 U of Phusion High-Fidelity DNA polymerase, 1X Phusion HF buffer and 50 ng DNA were used. The resulting PCR product was cloned into the pGEM-T Easy vector (Promega) and named pGEM-GGT1. The sequence of the cloned PCR-amplified fragment was confirmed, using M13 universal primers and internal sequencing primers (GGT1 seq 1 through 8, in Table 1) as indicated in Section 2.2.2.

2.2.5. Targeted gene replacement of CgGGT1

The 5′ (−1442 to +150 bp from the start codon) and 3′ (−149 to +1353 bp from the stop codon) flanking regions of *CgGGT1* were amplified with primer pairs GGT1 KO 5′ and GGT1 KO 3′, respectively. The PCR product of the 5′ region was digested with *Kpn*I and *Xho*I, and cloned into pCB1636, which contains the *hph* gene for hygromycin B resistance (Sweigard et al., 1997). Then, the PCR product of the 3′ region was digested with *Eco*RI and *Bam*HI, and ligated into the previous construct. The resulting vector, pGGT1Δ-hph, was digested with restriction enzymes and sequenced. The pGGT1Δ-hph vector was linearized with *Xba*I, and transformed into *C. graminicola* wild type protoplasted oval conidia by PEG-mediated transformation (Amnuaykanjanasin and Epstein, 2003; Epstein et al., 1998). Putative hygromycin-resistant transformants were single-spore purified, and then confirmed by PCR with hygromycin-specific primers HygB. To identify deletion mutants, 34 hygromycin-resistant transformants were screened by PCR with the internal *CgGGT1* primers GGT1 Seq4, which amplify the *CgGGT1* gene from wild type and ectopic transformants, but not knockouts (Fig. 1A). Two gene replacement mutants were confirmed by restriction analysis, Southern blot and sequencing.

2.2.6. Complementation of CgGGT1Δ and construction of over-expressing CgGGT1 strains

A complementation vector, pGGT1-bar, was constructed with the 4.2 kb fragment in pGEM-GGT1 (Section 2.2.4). The pGEM-GGT plasmid was digested with *Eco*RI, and the 4.2 kb fragment was purified and ligated into *Eco*RI-digested pCB1534 (Sweigard et al., 1997), which encodes the *bar* gene for resistance to glufosinate ammonium. The pGGT1-bar complementation vector 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, 0.5% ammonium sulfate, 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.

2.2.7. Southern blot hybridization analysis

The *CgGGT1* 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 pCB1636 with the Hyg 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 *Msc*I 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 (GpNA; 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 glycylglycine, 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/vogels.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 1×10^4 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 leaf 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 ($P < 0.05$), $\log_{10}$-transformed data were used to satisfy the assumption of homoscedasticity for ANOVA. Means separation was performed using Tukey's HSD ($\alpha = 0.05$). 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)(LIVMA)X_4G(SN)XV(STA)XTXT(LIVM)(NE)X_{1-2}(FY)G (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 TGANTN (Springael and Penninckx, 2003) and the algorithm at The *Saccharomyces cerevisiae* Promoter Database (http://rulai.cshl.edu/SCPD), potential GATA regulatory elements were identified in all three *CgGGT*s: *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 *CgGGT*s were transcribed in wild type conidia (data not shown), germlings (Fig. 2) and mycelia (Fig. 3A–C). Although the three *CgGGT*s were repeatedly detected in conidia by quantitative RT-PCR, levels of *CgGGT1* were too variable for meaningful inference (data not shown). In the wild type, nitrogen-starved germlings had significantly ($\alpha = 0.05$) more (4.5X) *CgGGT1* transcripts than those in nitrogen-rich media. Based on comparisons of the 95% confidence intervals (CI_{95}), the transcription of *CgGGT1* in the wild type was significantly greater than *CgGGT3* during nitrogen starvation.

To quantify expression of *CgGGT*s during plant infection, quantitative RT-PCR (Fig. 4) also was used to analyze infected tissue in the biotrophic phase 4 days post inoculation (dpi) when host tissue was asymptomatic, and in the necrotrophic phase (6 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 *GGT*s in the biotrophic versus the necrotrophic stage ($P_{CgGGT1} = 0.72$; $P_{CgGGT2} = 0.77$, $P_{CgGGT3} = 0.18$). Because *CgGGT1* expression was greater in media and *in planta*, it was chosen for further characterization.

3.3. Generation and selection of CgGGT1 knockouts, 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 ($CgGGT1^{OE}$) 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*

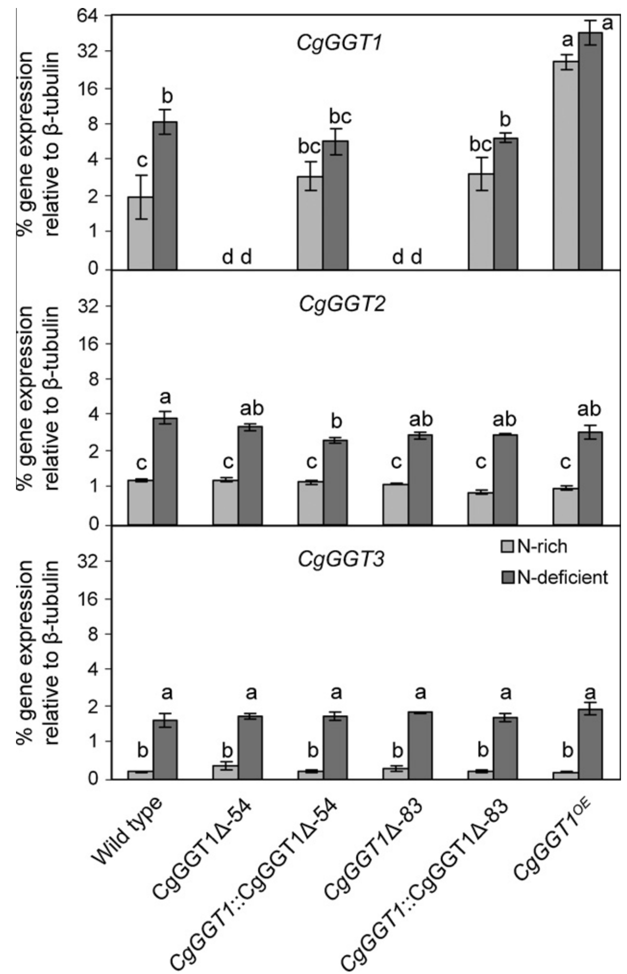

Fig. 2. mRNA expression in *C. graminicola* germlings of the three γ-glutamyltransferases (GGTs) in nitrogen-rich and nitrogen-deficient media. Petri dishes containing modified Fries medium with and without nitrogen were inoculated with conidia of wild type, *CgGGT1Δ*, complemented *CgGGT1Δ* and *CgGGT1*-over-expressing strains. After 12 h of growth, expression 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 $2^{-\Delta Ct}$ method. Data were $\log_{10}$-transformed to achieve homogeneity of variance, and are presented on a log scale. Means ± SE of three biological replicates are shown. Due to a significant strain ∗ nitrogen interaction in *CgGGT1*, Tukey's analysis is shown for a one-way analysis. For each panel, bars with the same letter are not significantly different by Tukey's HSD, $\alpha = 0.05$.

strains had wild-type levels of *CgGGT* transcription. The $CgGGT1^{OE}$ 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 $CgGGT1^{OE}$ 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, $CgGGT1^{OE}$, two *CgGGT1Δ*, and the two complemented *CgGGT1Δ* strains. However, based on

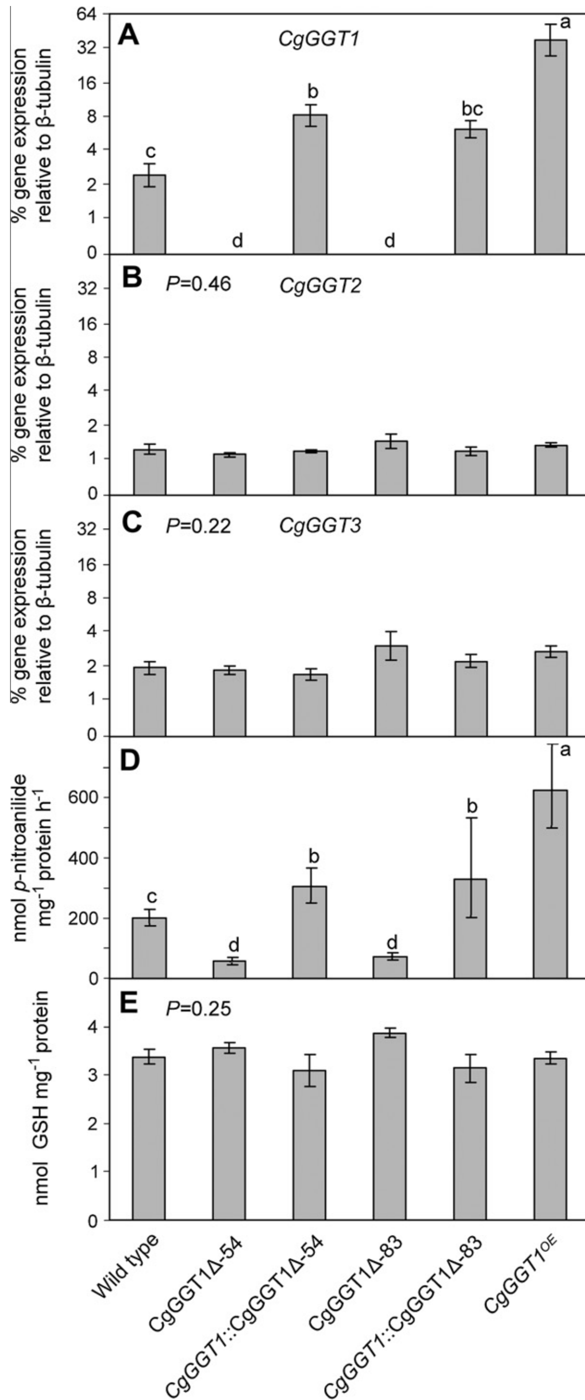

Fig. 3. mRNA expression of the three γ-glutamyltransferases (GGTs), GGT activity and glutathione (GSH) concentration in *C. graminicola* mycelia. Five-day-old mycelia from the wild type, the *CgGGT1*Δ mutants, the complemented *CgGGT1*Δ mutants and *CgGGT1*-over-expressing strain were collected from 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 $2^{-\Delta Ct}$ 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.

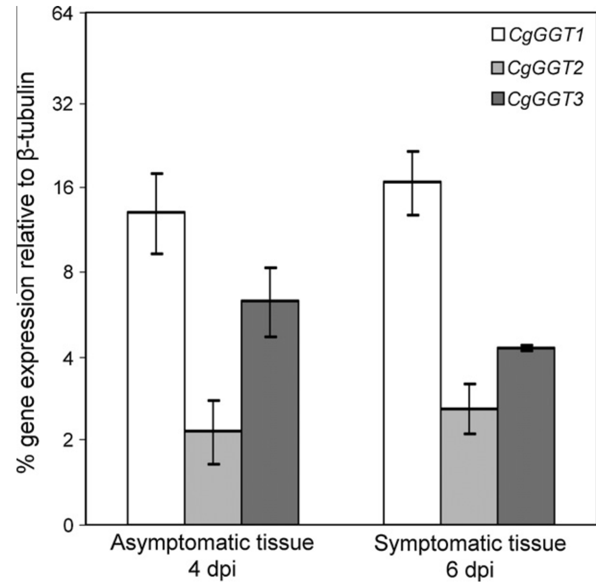

Fig. 4. mRNA expression of the three *C. graminicola* γ-glutamyltransferases (*GGT*s) *in planta.* Sheath leaves of seven-week-old highly susceptible corn cv. Mo940 were inoculated with a mycelial agar plug. Lesions were excised from sheaths 4 and 6 days post-inoculation (dpi). Expression analysis of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

CI_{95}, mycelial homogenates were a significantly richer source of GGT than conidial homogenates (compare Figs. 3D and 5A). For example, the GGT CI_{95} in the wild type was 18–41 and 175–230 nmol *p*-nitroanilide mg^{-1} protein h^{-1}, for the conidia and mycelia, respectively. Mycelia of the two *CgGGT1*Δ 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 *CgGGT1*Δ 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 ∗ 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 *CgGGT1*Δ strains and 314% ± 25% in the $CgGGT1^{OE}$ ($n = 4$) 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 *CgGGT* 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 $CgGGT1^{OE}$ 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 *CgGGT*s in germlings of the six strains in nitrogen-rich versus nitrogen-deficient media

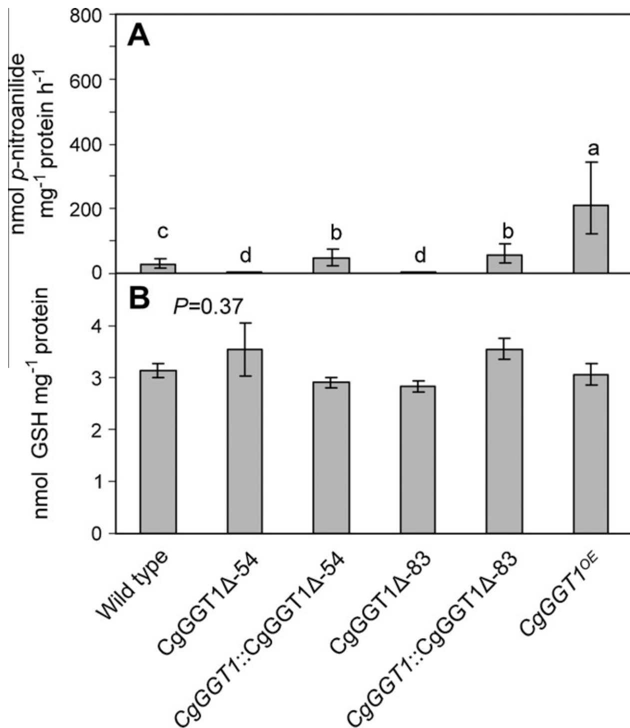

Fig. 5. γ-Glutamyltransferase (GGT) activity and glutathione (GSH) concentration in *C. graminicola* conidia. Falcate conidia from wild type, the *CgGGT1*Δ mutants, the complemented *CgGGT1*Δ mutants and *CgGGT1*-over-expressing strains were harvested from 7-day-old cultures on oatmeal agar. (A) GGT activity at pH 7.5 was measured using γ-glutamyl *p*-nitroanilide as donor and glycylglycine as acceptor substrate. GGT activity values were $\log_{10}$-transformed to achieve homoscedasticity. Detransformed means of four biological replicates and error bars with the 95% confidence intervals are shown. Bars with the same letter are not significantly different by Tukey's HSD, $\alpha = 0.05$. (B) GSH content was measured by HPLC with electrochemical detection. Means ± SE of three biological replicates are shown. There were no significant differences between strains.

indicated a significant ($P = 0.037$) nitrogen ∗ strain interaction for *CgGGT1*, but not for either *CgGGT2* or *CgGGT3* ($P > 0.05$). In all strains, nitrogen starvation significantly ($P < 0.0001$) induced expression of *CgGGT2* and *CgGGT3* in germlings (Fig. 2). In wild type germlings, nitrogen starvation significantly ($\alpha = 0.05$) increased *CgGGT1* transcription. However in strains with ectopic copies of *CgGGT1*, nitrogen starvation did not significantly increase transcription in germlings ($P > 0.05$; Fig. 2).

3.6. *C. graminicola CgGGT1 apparently enables utilization of GSH as a nitrogen source during nitrogen deficiency*

To determine whether GSH can be utilized for growth during nitrogen deficiency, the *CgGGT1* strains were grown in nitrogen-deficient Vogel's agar medium amended with either 0, 3 or 6 mM GSH as the nitrogen source (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 (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 *CgGGT1*Δ mutants grew less than the wild type, complemented *CgGGT1*Δ 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 (Table 2). When strains were grown in modified Vogel's broth amended with either 3 or 6 mM GSH, both *CgGGT1*Δ strains had significantly less biomass ($P < 0.0001$), and only 23% ± 2% and 41% ± 3% (CI_{95}, $n = 8$) of the growth of the wild type, respectively. The $CgGGT1^{OE}$ 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% (CI_{95}, $n = 4$) more biomass, respectively, compared with the wild type. These results suggest that 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. *CgGGT1 is not required for virulence of C. graminicola in a wounded leaf assay*

To determine whether *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 *C. graminicola* is a hemibiotroph, 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 (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 (and the other strains) did not develop symptoms by 10 dpi (data not shown), whereas the highly susceptible cv. Mo940 had approximately four times more symptomatic tissue than the moderately susceptible Yellow Jubilee (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 ($\alpha = 0.05$; Table 3). Thus, *CgGGT1* is not required for virulence of *C. graminicola* in wounded corn sheaths.

3.8. *Deletion and over-expression of CgGGT1 has no effect on glutathione levels and C. graminicola CgGGT1 does not appear to be involved in tolerance of oxidative stress*

There were no significant differences in GSH content (Fig. 6) in conidia ($P = 0.37$; Fig. 5B) or mycelia ($P = 0.25$; Fig. 3E) in any of the *C. graminicola* strains. Concentrations of GSH in conidia and mycelia were indistinguishable with the $CI_{95} = 2.6–3.7$ and 2.7–4.0 nmol GSH mg^{-1} protein in wild type conidia and mycelia, respectively.

Based on either percentage conidial germination or *in vitro* growth assays, the *C. graminicola GGT* strains did not have any differential sensitivity to oxidants (*tert*-butyl hydroperoxide, menadione, and toluidine blue and rose bengal in the light), the heavy metal cadmium chloride, xenobiotics (chlorothalonil and mancozeb), and Calcofluor white (data not shown).

4. Discussion

This is the first evidence that a specific GGT in a filamentous fungus, CgGGT1, enables utilization of GSH as a nitrogen source *in vitro*. Deletion or over-expression of *CgGGT1* has a significant effect on mycelial growth of *C. graminicola* in a nitrogen-deficient broth, in which GSH is the nitrogen source; in 3 mM GSH, the knockouts and over-expressing strains had approximately one-quarter and 3-fold the mycelial biomass, respectively, of the wild type. The results on agar in Supplemental Fig. 1 also indicate that 6 mM GSH

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

Strain	Nitrogen source in Vogel's modified broth		
	3 mM GSH[b]	6 mM GSH[b]	Standard 25 mM ammonium nitrate
	Mycelia dry weight (mg ± SE) per dL broth[c]		
Wild type	104 ± 4 c	97 ± 3 b	255 ± 14 a
CgGGT1Δ-54	27 ± 2 d	46 ± 3 c	243 ± 20 ab
CgGGT1Δ-83	22 ± 2 d	40 ± 2 c	238 ± 6 ab
CgGGT1::CgGGT1Δ-54	139 ± 7 b	122 ± 11 b	186 ± 10 b
CgGGT1::CgGGT1Δ-83	122 ± 4 bc	121 ± 5 b	207 ± 16 ab
CgGGT1 over-expressing	284 ± 7 a	293 ± 12 a	197 ± 10 ab

[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[a].

Strain	Cultivar	
	Highly susceptible cv. Mo940	Moderately susceptible cv. Yellow Jubilee
	Symptomatic area (% ± SE)[b]	
Wild type	10.1 ± 0.6 a	2.5 ± 0.2 a
CgGGT1Δ-54	9.5 ± 0.8 a	3.0 ± 0.3 a
CgGGT1Δ-83	11.6 ± 0.6 a	3.0 ± 0.3 a
CgGGT1::CgGGT1Δ-54	9.9 ± 0.7 a	3.0 ± 0.3 a
CgGGT1::CgGGT1Δ-83	11.2 ± 0.5 a	2.9 ± 0.3 a
CgGGT1 over-expressing	11.8 ± 0.5 a	2.7 ± 0.3 a
Mock-inoculated control	0.2 ± 0.2 b	0.0 ± 0.0 b

[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 Leotiomycete 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

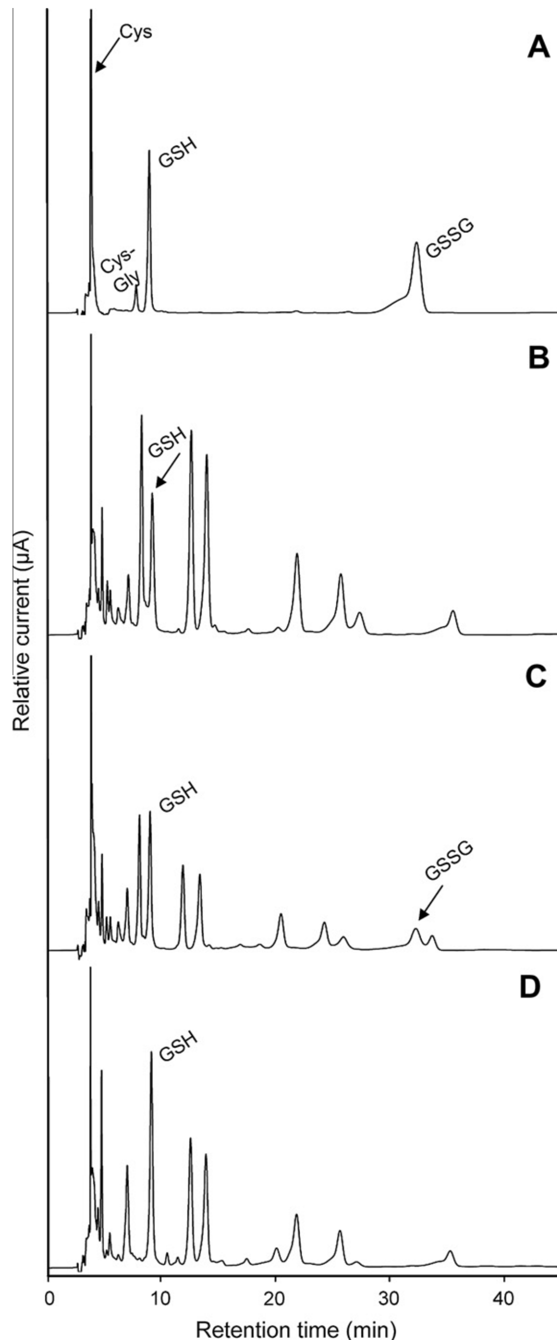

Fig. 6. Chromatogram of reverse phase HPLC separation of 10% perchloric acid-soluble extracts with electrochemical detection. Chromatogram (A) of low-molecular weight thiol standards: 25 pmol of each of cysteine (cys; 3.9 min); cysteinyl-glycine (cys-gly; 7.8 min); reduced glutathione (GSH; 8.9 min); 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.

C. graminicola wild type and *CgGGT1*Δ 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 Cg*GGT1* 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 (Ubiyvovk et al., 2006), and one that is closer to physiological pH.

Estimates of GSH concentration with EC detection were reasonably precise, with 95% confidence intervals from 170 to 421 ng GSH mg^{-1} dry weight in wild type conidia and 173 to 273 ng GSH mg^{-1} dry weight in mycelia. Expressed as means + SE, there were similar levels of GSH in 7 day-old conidia produced on oatmeal agar (206 ± 37 ng GSH mg^{-1} dry weight) and 5 day-old mycelia grown in Fries medium (215 ± 37 ng GSH mg^{-1} dry weight). GSSG was not detected in either conidia or mycelia.

Although GGT activity in the over-expressed and knockouts were significantly increased and decreased, respectively, GSH levels were unaffected ($P > 0.05$) in conidia, germlings and mycelia. GSH levels were decreased in nitrogen-starved germlings, but levels were indistinguishable between the *CgGGT1*Δ and wild type strains. In contrast, the *S. sclerotiorum Ss-ggt1* knockout had a 10-fold higher concentration of total glutathione in sclerotia than the wild type (Li et al., 2012). Similarly, when either *GGT1* or *GGT2* in *S. pombe* were over-expressed, mutant cells had significantly higher levels of GSH than the wild type cells (Park et al., 2005, 2004). However, in *S. cerevisiae*, *CIS2*Δ cells had significantly lower concentrations of GSH than the wild type (Gales et al., 2008); for example, GSH concentration during the exponential growth phase in *CIS2*Δ and wild type strains was 4 and 11 mM, respectively.

In *S. sclerotiorum*, *SsGgt1*Δ strains over-produced sclerotial initials, and produced malformed sclerotia with aberrant rinds that failed to carpogenically produce apothecia (Li et al., 2012). In contrast, other than differential growth in GSH, no phenotypes of the *CgGGT1*Δ and over-expressing strain were observed. There were no differences in colony morphology among all strains on oatmeal agar (used for sporulation) or PDA (used for vegetative growth). The *CgGGT1* mutant strains and the wild type both formed appressoria on glass slides and on detached corn leaves (data not shown). The *C. graminicola* GGT strains did not have any differential sensitivity to oxidants (*tert*-butyl hydroperoxide, menadione, and toluidine blue and rose bengal in the light), the heavy metal cadmium chloride and other xenobiotics (chlorothalonil and mancozeb). Consequently, there is no evidence that CgGGT1 is involved in tolerance of oxidative stress, in contrast to *S. cerevisiae* (Adamis et al., 2007, 2009; Grant et al., 1996; Santos et al., 2009; Shin et al., 2003; Ubiyvovk et al., 2006). Differences in phenotypes between the

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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