Clades of γ-Glutamyltransferases (GGTs) in the Ascomycota and Heterologous Expression of *Colletotrichum graminicola* CgGGT1, a Member of the Pezizomycotina-only GGT Clade

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Gamma-glutamyltransferase (GGT, EC 2.3.2.2) cleaves the γ-glutamyl linkage in glutathione (GSH). Ascomycetes in either the Saccharomycotina or the Taphrinomycotina have one to three GGTs, whereas members of the Pezizomycotina have two to four GGTs. A Bayesian analysis indicates there are three well-supported main clades of GGTs in the Ascomycota. 1) A Saccharomycotina and a Taphrinomycotina-specific GGT sub-clade form a yeast main clade. This clade has the three relatively well-characterized fungal GGTs: (*Saccharomyces cerevisiae* CIS2 and *Schizosaccharomyces pombe* Ggt1 and Ggt2) and most of its members have all 14 of the highly conserved and critical amino acids that are found in GGTs in the other kingdoms. 2) In contrast, a main clade (GGT3) differs in 11 of the 14 highly conserved amino acids that are found in GGTs in the other kingdoms. All of the 44 Pezizomycotina analyzed have either one or two GGT3s. 3) There is a Pezizomycotina-only GGT clade that has two well-supported sub-clades (GGT1 and GGT2); this clade differs in only two of the 14 highly conserved amino acids found in GGTs in the other kingdoms. Because the Pezizomycotina GGTs differ in apparently critical amino acids from the cross-kingdom consensus, a putative GGT from *Colletotrichum graminicola*, a member of the Pezizomycotina, was cloned and the protein product was expressed as a secreted protein in *Pichia pastoris*. A GGT enzyme assay of the *P. pastoris* supernatant showed that the recombinant protein was active, thereby demonstrating that CgGGT1 is a *bona fide* GGT.

Keywords: EC 2.3.2.2, gamma-glutamyltransferase, gamma-glutamyltranspeptidase, glutathione catabolism, glutathione utilization

Introduction

Glutathione (GSH; L-γ-glutamyl-L-cysteinyl-glycine) is a tripeptide that is present in high concentration in eukaryotic cells. In *Saccharomyces cerevisiae*, GSH can comprise 1% of the dry weight (Meister and Anderson, 1983; Pócsi *et al.*, 2004). GSH is essential for cell viability, and deletion of the γ-glutamyl-cysteine ligase gene, which encodes for the first enzyme in GSH biosynthesis, is embryo-lethal in mouse and Arabidopsis (Shi *et al.*, 2000; Cairns *et al.*, 2006). In *S. cerevisiae* and *Schizosaccharomyces pombe*, mutants that lack γ-glutamyl-cysteine ligase are unable to grow in minimal media unless exogenous GSH is added (Grant *et al.*, 1996; Chaudhuri *et al.*, 1997). GSH plays a role in thiol-redox maintenance (Penninckx and Elskens, 1993; Pócsi *et al.*, 2004), in cytosolic iron metabolism (Kumar *et al.*, 2011), and may serve as a nitrogen source during nitrogen and sulfur starvation in *S. cerevisiae* (Elskens *et al.*, 1991; Mehdi and Penninckx, 1997; Kumar *et al.*, 2003) and in *S. pombe* (Song and Lim, 2008).

GSH has an unusual γ-glutamyl peptide bond, which makes it resistant to degradation by most peptidases (Penninckx and Elskens, 1993; Pócsi *et al.*, 2004). γ-Glutamyltransferase (GGT, EC 2.3.2.2, synonym γ-glutamyltranspeptidase) cleaves the γ-glutamyl linkage in GSH. GGTs are evolutionary conserved and have had lineage-specific expansion in eukaryotes. Bacterial organisms such as *Escherichia coli* have a single GGT gene (Suzuki *et al.*, 1988). Amongst the higher eukaryotic organisms, *Arabidopsis thaliana* has four GGT genes, although one may be a pseudogene (Martin *et al.*, 2007). The human GGT family has thirteen homologs, but only six may be transcribed. In contrast, the rat genome has a single GGT copy, but five promoters and alternative splicing generates seven transcripts (Zhang and Forman, 2009).

Almost all knowledge of GGT in fungi is from studies of either *S. cerevisiae* (Penninckx and Elskens, 1993; Mehdi *et al.*, 2001; Kumar *et al.*, 2011), which is in the Saccharomycotina and has one GGT or *S. pombe* (Kang *et al.*, 2005; Kim *et al.*, 2005; Park *et al.*, 2005), which is in the Taphrinomycotina and has two GGTs. However, the largest subphylum of the Ascomycota is the Pezizomycotina, which contains the filamentous ascomycetes, and the majority of the plant pathogens.

In mammalian cells, GSH is only cleaved by GGT. In *S. cerevisiae*, which has only one GGT, called CIS2, vacuolar GSH is cleaved by the GGT CIS2, which is in the vacuole membrane. In *S. pombe*, which has two GGTs, Ggt1 is localized in the vacuolar membrane and Ggt2 is localized in the endoplasmic reticulum (Jasper and Penninckx, 1984; Matsuyama *et al.*, 2006). The *S. pombe* Ggt1 knockout is lethal but knockouts in either *S. cerevisiae* CIS2 or in *S. pombe* Ggt2 are viable (Kim *et al.*, 2010). *S. cerevisiae* has an alternative non-GGT mediated DUG pathway for GSH cleavage that was elucidated recently (Kaur *et al.*, 2012). Cytoplasmic

Table 1. The number of γ-glutamyltransferases (GGTs) and the GGT clades in the selected full-genome-sequenced fungi in this study[a]

Species	Phylum, subphylum or class[b]	No. GGTs	GGT clades[c]
Alternaria brassicicola	Dothidiomycetes	2	GGT1, GGT3
Ashbya gossypii	Saccharomycotina WDG[f]	1	GGT-S/T
Aspergillus clavatus[d]	Eurotiomycetes	2	GGT1, GGT3
Aspergillus flavus[d,e]	Eurotiomycetes	4	3GGT1, GGT3
Aspergillus fumigatus	Eurotiomycetes	2	GGT1, GGT3
Aspergillus nidulans	Eurotiomycetes	2	GGT1, GGT3
Aspergillus niger[d]	Eurotiomycetes	2	GGT1, GGT3
Aspergillus oryzae	Eurotiomycetes	4	Three GGT1, GGT3
Aspergillus terreus[d]	Eurotiomycetes	2	GGT1, GGT3
Botrytis cinerea	Leotiomycetes	3	GGT1, GGT2, GGT3
Candida albicans	Saccharomycotina CTG[g]	2	GGT3S, GGT-S/T
Candida glabrata	Saccharomycotina WDG	1	GGT-S/T
Candida guillermondi	Saccharomycotina CTG	2	GGT3S, GGT-S/T
Cercospora zea maydis	Dothidiomycetes	2	GGT1, GGT3
Coccidioides immitis	Eurotiomycetes	2	GGT1, GGT3
Cochliobolus heterostrophus	Dothidiomycetes	2	GGT1, GGT3
Cochliobolus sativus	Dothidiomycetes	2	GGT1, GGT3
Colletotrichum graminicola	Sordariomycetes	3	GGT1, GGT2, GGT3
Colletotrichum higginsianum	Sordariomycetes	3	GGT1, GGT2, GGT3
Debaromyces hansenni	Saccharomycotina CTG	3	Two GGT3S, GGT-S/T
Dothistroma septosporum	Dothidiomycetes	2	GGT1, GGT3
Encephalitozoon intestinalis	Microsporidia	1	(Outgroup)
Fusarium graminerarum	Sordariomycetes	3	GGT1, GGT2, GGT3
Fusarium oxysporum	Sordariomycetes	3	GGT1, GGT2, GGT3
Fusarium verticillioides	Sordariomycetes	3	GGT1, GGT2, GGT3
Histoplasma capsulatum	Eurotiomycetes	2	GGT1, GGT3
Kluveromyces lactis	Saccharomycotina WDG	2	GGT3S, GGT-S/T
Leptosphaeria maculans	Dothidiomycetes	2	GGT1, GGT3
Magnaporthe grisea	Sordariomycetes	3	GGT1, GGT2, GGT3
Microsporum canis	Eurotiomycetes	3	Two GGT1, GGT3
Microsporum gypseum	Eurotiomycetes	4	Two GGT1, GGT3, GGT-Pezizo
Mycosphaerella fijiensis	Dothidiomycetes	3	GGT1, GGT3, GGT-Pezizo
Mycosphaerella graminicola	Dothidiomycetes	2	GGT1, GGT3
Nectria haematococca	Sordariomycetes	3	GGT1, GGT2, GGT3
Neosartorya fischeri[d]	Eurotiomycetes	2	GGT1, GGT3
Neurospora crassa	Sordariomycetes	2	GGT2, GGT3
Neurospora discreta	Sordariomycetes	2	GGT2, GGT3
Neurospora tetrasperma	Sordariomycetes	2	GGT2, GGT3
Oidiodendron maius	Leotiomycetes	4	GGT1, GGT2, GGT3, GGT-Pezizo
Paracoccidioides brasiliensis	Eurotiomycetes	2	GGT1, GGT3
Pyrenophora teres	Dothidiomycetes	2	GGT1, GGT3
Pyrenophora tritici-repentis	Dothidiomycetes	2	GGT1, GGT3
Saccharomyces cerevisiae	Saccharomycotina WDG	1	GGT-S/T
Saccharomyces kluyveri	Saccharomycotina WDG	2	GGT3S, GGT-S/T
Schizosaccharomyces japonicus	Taphrinomycotina	2	Two GGT-S/T
Schizosaccharomyces octosporus	Taphrinomycotina	2	Two GGT-S/T
Schizosaccharomyces pombe	Taphrinomycotina	2	Two GGT-S/T
Sclerotinia sclerotiorum	Leotiomycetes	3	GGT1, GGT2, GGT3
Setosphaeria turcica	Dothidiomycetes	2	GGT1, GGT3
Stagonospora nodorum	Dothidiomycetes	2	GGT1, GGT3
Trichoderma atroviridae	Sordariomycetes	4	GGT1, GGT2, GGT3, GGT3-Tricho
Trichoderma reesei	Sordariomycetes	4	GGT1, GGT2, GGT3, GGT3-Tricho
Trichoderma virens	Sordariomycetes	4	GGT1, GGT2, GGT3, GGT3-Tricho
Trichophyton rubrum	Eurotiomycetes	3	GGT1, GGT2, GGT3
Verticillium albo-atrum	Sordariomycetes	3	GGT1, GGT2, GGT3
Verticillium dahliae	Sordariomycetes	3	GGT1, GGT2, GGT3
Yarrowia lypolitica	Saccharomycotina WDG	2	GGT3S, GGT-S/T

[a] All of the fungi are in the Phylum Ascomycota, except for the outgroup *Encephalitozoon intestinalis*, which is in the Microsporidia.
[b] The Saccharomycotina and Taphrinomycotina are subphyla. The Dothidiomycetes, Eurotiomycetes, Leotiomycetes, and Sodariomyces are classes within the subphylum Pezizomycotina.
[c] GGT clades determined in this study are shown in Fig. 1. GGT1, a Pezizomycotina-only sub-clade within the Pezizomycotina clade; GGT2, a Pezizomycotina-only sub-clade within the Pezizomycotina clade; GGT-Pezizo, members of the Pezizomycotina only clade that are neither in GGT1 nor GGT2; GGT-S/T, a clade containing one sub-clade with only Saccharomycotina and another sub-clade with only Taphrinomycotina; GGT3, a clade that includes a Pezizomycotina and a Saccharomycotina sub-clade (GGT3-S); GGT3-Tricho, a GGT3 sub-clade that is specific to *Trichoderma* spp.
[d] These species were used for the expanded *Aspergillus* spp. GGT analysis in Fig. 2, but not in the analysis in Fig. 1.
[e] Two different *Aspergillus flavus* strains were included in the analysis in Fig. 2.
[f] Saccharomycotina WGD, yeasts that have undergone whole-genome duplication.
[g] Saccharomycotina CTG, yeasts that translate the CUG codon into serine instead of leucine

GSH is degraded by the cytosolic Dug complex (Baudouin-Cornu *et al.*, 2012). DUG2p and DUG3p form a glutamine amidotransferase (GATase) that cleaves the γ-glutamyl bond. The dipeptidase Dug1p then cleaves the cysteinyl-glycine (Kaur *et al.*, 2009). Although the DUG pathway has not been confirmed in other fungi, the filamentous fungus *Colletotrichum graminicola*, for example, has homologs with an $E_{(Expect\ value\ of\ a\ random\ match)} = 0.0$ (Karlin and Altschul, 1990) of
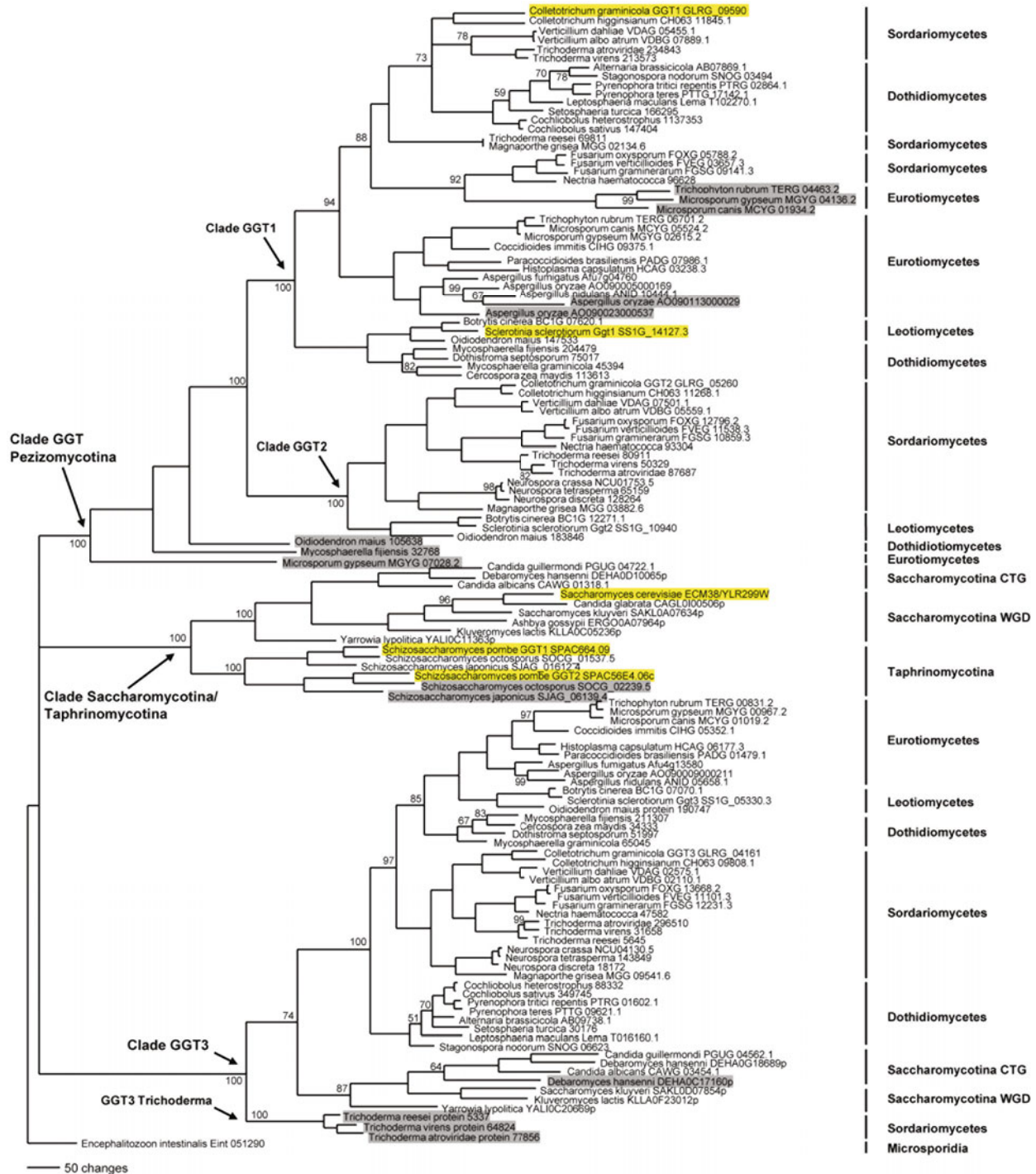

Fig. 1. The evolution of γ-glutamyl transferases (GGTs) from the Ascomycota inferred by Bayesian analysis. Fifty one fungal species, and one microsporidian (used as an outgroup), were included in the analysis. Species name are followed by their accession numbers. Numbers on the nodes indicate posterior probabilities of the 50% majority-rule consensus tree estimated from 2,942 trees. Because most nodes had 100% support values, only support values less than 100% and the 100% values for nodes of interest are shown. Taxonomic groups are indicated on the right. Saccharomycotina WGD are yeasts that have undergone whole-genome duplication. Saccharomycotina CTG are yeasts that translate the CUG codon into serine instead of leucine. Functionally characterized GGTs are highlighted in yellow. Putatively duplicated GGTs are shadowed in gray.

all three *S. cerevisiae* DUG proteins. Consequently, it is highly likely that filamentous fungi have the DUG pathway.

Sclerotinia sclerotiorum, a Leotiomycete in the Pezizomycota, is the only filamentous fungus in which there has been a mutational analysis of a GGT (Li *et al.*, 2012). Li *et al.* (2012) characterized *Ss-Ggt1* and reported that expression of *Ss-Ggt1* is developmentally dependent and is expressed more during development of sclerotia, apothecia and compound appressoria. Deletion mutants of *Ss-Ggt1* (*Ss-Ggt1Δ*) produced defective compound appressoria and aberrant sclerotia that did not develop normally and did not produce apothecia. The deletion mutants also had more total GSH and peroxide than the wild type. The *Ss-Ggt1Δ* mutants were less virulent on host leaves than the wild type, but only when inoculated onto unwounded rather than wounded tomato leaves.

Here, we present the first *in silico* examination of the evolution of γ-glutamyltransferases in the Ascomycota. We demonstrate that there are three well-supported main clades: a Saccharomycotina/Taphrinomycotina-only clade that contains all of the well characterized yeast GGTs and has highly conserved critical sites; a previously unrecognized GGT3 clade that has been conserved within the Pezizomycotina and some Saccharromycotina, but is highly divergent from the cross-kingdom consensus of critical residues; and a previously unrecognized Pezizomycotina-only clade that contains a duplication in the Sodariomycete/Leotiomycete lineage. Furthermore, we cloned and expressed a *C. graminicola* GGT (GLRG_09590, *CgGGT1*) from the Pezizomycotina-only clade and demonstrated that the transformed *Pichia pastoris* secretes a *bona fide* GGT with γ-glutamyltransferase activity.

Materials and Methods

The evolution of fungal GGTs

Fifty one full genome-sequenced ascomycetes were originally selected as representatives of the Pezizomycotina (15 Sodariomycetes, 3 Leotiomycetes, 9 Eurotiomycetes, and 12 Dothidiomycetes), 9 Saccharomycotina, and 3 Taphrinomycotina (Fig. 1 and Table 1). *Encephalitozoon intestinalis* in the Microsporidia was selected as the outgroup because the Microsporidia have a single GGT and may be the basal lineage for fungi (Corradi and Slamovits, 2011). Using *C. graminicola* GGT1 as the query, GGT sequences were obtained from either the Broad Institute Fungal Genome Initiative (www.broadinstitute.org), the DOE Joint Genome Initiative Fungal Genomics Program (genome.jgi-psf.org/programs/fungi/index.jsf), or the Génolevures Consortium (www.genolevures.org). Putative GGTs were confirmed with the NCBI CDD conserved domain database (Marchler-Bauer *et al.*, 2011). GGT sequences were trimmed at the 5′ and 3′ ends to remove portions that had no homology to the GGT domain region; each trimmed sequence had 780 characters, i.e., amino acids and gaps. Sequences were aligned using ClustalW 2.0 (Larkin *et al.*, 2007) and edited manually.

A Bayesian phylogenetic analysis was performed with MrBayes v3.1.2 (Ronquist and Huelsenbeck, 2003). Two analyses with 500,000 generations were run in parallel, each with four chains, and sampling every 100 generations. A gamma-shaped distribution of rates and a mixed prior probability for amino acid substitution was used. The 2,942 (2×1,471) sampled trees that converged with an average standard deviation of split frequencies <0.040 were selected and branch length and posterior probabilities for each node of a 50% majority-rule consensus tree were estimated using PAUP 4.0b (Swofford, 2003). An additional analysis was conducted on all full genome-sequenced *Aspergillus* spp. (*Aspergillus* Genome Database, www.aspergillusgenome.org), including the three *Aspergillus* spp. (*A. fumigatus*, *A. nidulans*, and *A. oryzae*) in the original analysis, and four additional *Aspergillus* species (*A. clavatus*, two isolates of *A. flavus*, *A. niger*, and *A. terreus*).

Colletotrichum graminicola culture and RNA isolation

Colletotrichum graminicola wild type isolate M1.001, the sequenced strain, was maintained in silica gel at -70°C. To produce falcate conidia, cultures were seeded onto Petri dishes with oatmeal agar (30 g oatmeal flour and 20 g agar per L) and incubated in constant light at 24 to 26°C for 7 days. Conidia were collected in ice-cold water, washed by centrifugation, and then lyophilized.

Ten mg of lyophilized conidia were ground manually in a 1.5 ml microfuge tube with 250 μl of 0.5 mm diameter glass beads (BioSpec, USA) and a metal pestle. Total RNA was isolated using the RNeasy Plant Mini kit (Qiagen, USA) according to manufacturer's recommendations. cDNA was synthesized from 1.5 μg of total RNA using MMLV reverse transcriptase (Promega, USA) with an oligo(dT)$_{20}$ primer, following the manufacturer's recommendations. Purified RNA was subsequently treated with DNase using the TURBO DNA-*free* kit (Ambion, USA). The integrity and yield of RNA was determined by gel electrophoresis and spectrophotometric analysis using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, USA).

Heterologous expression of CgGGT1 in *P. pastoris* as a secreted protein

The following *CgGGT1* cDNA (GenBank JQ413977) primers were designed with the deletion of the start codon and the last four nucleotides, and with additional restriction sites, shown as bolded and underlined bases: forward, 5′-TTTT**GAATTC**AAGCTCTCCTTGACTTCTGCT-3′; and reverse, 5′-GGG**TCTAGA**ACAGCAAATCCTCCCGAGT-3′. After PCR products were purified with the QIAquick PCR purification kit (Qiagen), the amplicon was digested with *Eco*RI and *Xba*I, and cloned in-frame into the pPICZαA vector with the *S. cerevisiae* α-factor secretion signal under the control of the *AOX1* methanol-inducible promoter, following the protocol of the EasySelect Pichia Expression Kit manual (Invitrogen, USA). The expressed protein also had a C-terminal peptide containing the *c-myc* epitope and polyhistidine tag. The resulting plasmid (pPICZαA-*GGT1*) and the empty vector were linearized with *Sac*I and electroporated into KM71H *P. pastoris* cells using a Gene Pulser Xcell System (Bio-Rad, USA). Transformants were selected for resistance to zeocin (100 μg/ml) in YPD with 1 M sorbitol (YPDS) media.

Genomic DNA was isolated from manually-ground lyophi-

lized *Pichia* cells using the DNeasy Plant Mini kit (Qiagen) according to the manufacturer's recommendations. After DNA elution, 10 μl (10 mg/ml) RNase A (Fermentas, USA) was added to the purified genomic DNA and the tube was incubated at 65°C for 1 h. Ten randomly selected zeocin-resistant colonies were analyzed by PCR with primers α-Factor (5′-TACTATTGCCAGCATTGCTGC-3′) and 3′-AOX1 (5′-GCAAATGGCATTCTGACATCC-3′) according to manufacturer's recommendations (Invitrogen) to confirm the integration of *CgGGT1*. Transformation of three zeocin-resistant and PCR-positive colonies was further confirmed by sequencing.

Expression and secretion of recombinant CgGGT1 into the culture media was demonstrated first by Western blot analysis, and then by an enzyme assay. For the Western, a 125 ml flask with 10 ml of buffered complex medium containing glycerol (BMGY) was inoculated with a single colony, and incubated at 30°C overnight at 250 rpm on a rotary shaker. Cells were harvested by centrifugation and the supernatant removed. The pellet was resuspended in 10 ml of buffered complex medium containing methanol (BMMY) to induce protein expression. Flasks incubated at 30°C were shaken at 250 rpm for 8 d. Every 24 h, to maintain induction, 100% methanol was added to the culture to a final concentration of 1.5%. After induction, 2.5 ml samples of culture supernatant were concentrated 10-fold in a Centricon-10 filter (Millipore, USA), and 20 μl of the concentrated sample were fractionated by SDS-PAGE using 10% Tris-HCl Ready Gel (Bio-Rad). Proteins were transferred to Immobilon (Millipore) PVDF membrane with a tank transfer system following manufacturer's instructions. The membrane was incubated with an anti-*c-myc*-peroxidase mouse monoclonal antibody according to the manufacturer's manual (Roche, USA). The chemiluminescent detection of the recombinant CgGGT1-tagged protein was performed using the Lumi-Light Western Blotting kit (Roche).

Quantification of γ-glutamyltransferase (GGT) activity and soluble protein

The γ-glutamyltransferase activity was quantified using the protocol of Tate and Meister (1985) with γ-glutamyl-*p*-nitro-anilide (GpNA; Sigma-Aldrich, USA) as the substrate molecule and glycylglycine (Sigma-Aldrich) as the donor molecule; the assay is frequently used with fungi (e.g., Ubiyvovk *et al.*, 2006). In preliminary experiments, we confirmed that the assay was linear in the detection range, i.e., increases in the concentration of the homogenate resulted in proportional increases in the release of *p*-nitroanilide. Briefly, the GGT activity assay was carried out in a volume of 750 μl consisting of 100 μl of cell-free *Pichia* BMMY culture supernatant, 100 mM Tris (pH 7.5), 10 mM EDTA, 20 mM glycylglycine, and either 2.5 mM GpNA or no GpNA (as a negative control for each treatment). Samples were incubated for 2 h on a tube rotator at 25°C. Afterwards, 50 μl of dichloromethane (DCM; Sigma-Aldrich) 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*-nitroanilide from GpNA was measured with a spectrophotometer (Ge-

nesys 10vis; Thermo Fisher Scientific, USA) by absorbance at 410 nm; GpNA-free controls were used as blanks for each sample.

To normalize GGT activity as nmol of secreted *p*-nitroanilide activity/mg/soluble protein in the cells/h, the pellet from 100 μl of *Pichia* BMMY culture was lyophilized, ground manually as indicated above, and suspended in 500 μl of TE buffer. After incubation at 37°C for 30 min to solubilize the protein, duplicate 50 μl samples were then assayed for soluble protein by the BCA method (Thermo Scientific Pierce) using 1 ml of working reagent and bovine serum albumin (BSA) as a standard. After incubation of the BCA reaction for 30 min at 37°C, 20 μl of DCM was added and the samples were vortexed and centrifuged. Protein levels in supernatants were determined spectrophotometrically at 562 nm. For GGT activity and protein assays, there were three in-
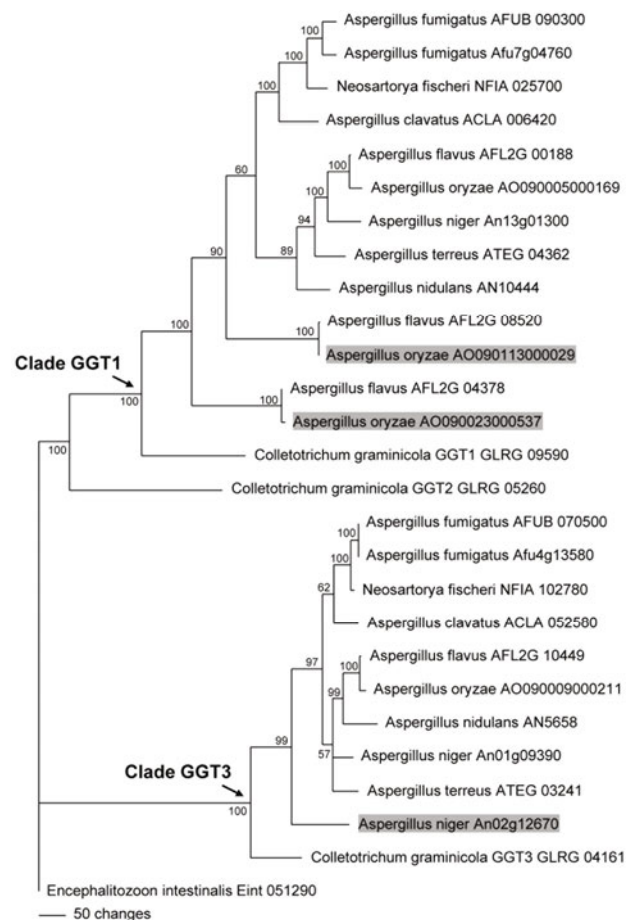

Fig. 2. The evolution of *Aspergillus* γ-glutamyl transferases (GGTs) inferred by Bayesian analysis. GGT sequences from full-genome sequenced *Aspergillus* species were obtained from the *Aspergillus* Genome Database (www.aspergillusgenome.org). The analysis includes two isolates of *A. fumigatus* (AFUB and Afu4g). *A. flavus* and *A. oryzae* are considered to be either closely related species or ecotypes of the same species (Payne *et al.*, 2006). *C. graminicola* GGTs were used as reference GGT clades, and the microsporidian *E. intestinalis* was used as an outgroup. Species name are followed by the gene accession number. Numbers on the nodes indicate posterior probabilities of the 50% majority-rule consensus tree estimated from 1,900 trees. Putatively duplicated GGTs are shadowed in gray.

dependent biological replicates for each treatment of supernatant from *Pichia* cells, with each replicate conducted as a separate trial.

Results and Discussion

The evolution of fungal GGTs

A phylogenetic analysis of GGTs in the Ascomycota revealed that there are three major, well-supported GGT clades. There is a Saccharomycotina/Taphrinomycotina-specific major clade comprised of a Saccharomycotina and a Taphrinomycotina sub-clade (Fig. 1). All nine of the Saccharomycotina have one member in this clade and all three of the Taphrinomycotina have two GGTs in this clade (Table 1). The sampled Taphrinomycotina only have GGTs in this clade, i.e., there was an ancient GGT duplication in this sub-clade. All of the Saccharomycotina have a single GGT in this clade and six of the nine have either one or two GGTs in another clade, GGT3. The Saccharomycotina/Taphrinomycotina-specific clade includes almost all of the previously studied fungal GGTs: *S. cerevisiae* CIS2, *S. pombe* Ggt1 and Ggt2 (Kang *et al.*, 2005; Kim *et al.*, 2005; Park *et al.*, 2005; Song and Lim, 2008), and *Hansenula polymorpha* GGT1 (Ubiyvovk *et al.*, 2006).

The major GGT3 clade contains at least one GGT of each of the 39 selected Pezizomycotina (Fig. 1). The GGT3 major clade has three sub-clades. The Pezizomycotina sub-clade includes one member in each Pezizomycotina, including in the three *Trichoderma* spp. There is also a *Trichoderma*-specific sub-clade that has an additional GGT3 in each of the three *Trichoderma* spp. Thus, there was a GTT3 duplication in an ancestral *Trichoderma* spp. The Saccharomycotina GTT3 sub-clade contains zero to two GGTs in the Saccharomycotina.

The Pezizomycotina-specific clade contains one to three GGTs in each of the 39 selected species (Fig. 1 and Table 1). The main clade has two well-supported two sub-clades: a GGT1 clade with zero to three GGTs in the 39 Pezizomycotina; a Sodariomycete/Leotiomycete-specific GGT2 sub-clade that has one GGT in each relevant species; and three GGTs that are neither in GGT1 nor GGT2. All three of the GGTs that are neither in GGT1 nor GGT2 are duplicates; the Leiotiomycete *Oidiodendron maius* has a duplicate in GGT1 and GGT2 and the Dothiodiomycetes *Mycosphaerella fijiensis* and *Microsporum gypseum* have duplicates in GGT1. There were other duplications within GGT1, including a duplication in a clade containing *Trichophyton rubrum*, *Microsporum gypseum*, and *Microsporum canis*, which are all members of the mitosporic Arthrodermataceae within the Eurotiomycetes. The GGT1 in *Aspergillus oryzae* was duplicated twice for a total of three GTT1s.

In order to further study the expansion GGTs in the Pezizomycotina, we performed an additional analysis of seven *Aspergillus* spp. (Fig. 2). We supported the evidence that *Aspergillus oryzae* had two duplications in GGT1 by demonstrating that the related *A. flavus* (Payne *et al.*, 2006) similarly has three copies of GGT1. Of the seven *Aspergillus* spp., *A. niger* had a duplication in GGT3.

An alignment of the deduced amino acid sequence of fungal (*S. cerevisiae*, *S. pombe*, and *C. graminicola*), bacterial (*Escherichia coli*), plant (*Arabidopsis thaliana*), and mammalian (human, and rat) GGTs shows that GGTs are evolutionary conserved (Fig. 3) with 29 strictly conserved amino acids. We compared residues in the fungal GGT clades at the four amino acid positions that are critical for GGT activity in both human GGT1 (Ikeda *et al.*, 1993, 1995a, 1995b, 1996; prosite.expasy.org/PDOC00404), and *E. coli* GGT (Okada *et al.*, 2006), an additional four positions that are critical for human GGT1, and an additional six positions that are critical for *E. coli* (Tables 2–4). Amino acid substitutions in those positions are shown in Fig. 3. Of the fungal GGTs, the Saccharomycotina/Tapphrinomycotina-specific clade (Table 2) is the most conserved with the cross-kingdom consensus, and most of its members have all 14 of the highly conserved and critical amino acids that are in GGTs in the other kingdoms. The Pezizomycotina-only GGT clade with GGT 1 and GGT2 (Table 3) has some diversity; with marked diversity in only two of the 14 analyzed positions. As expected there are examples of greater diversity in duplicated genes. For example, the *Trichophyton/Microsporum* GGT1 duplicated clade (*M. canis* MCYG 01934.2, *M. gypseum* MGYG 04136.2 and *T. rubrum* TERG 04463.2) diverge in 7 of the 14 position whereas the presumed ancestral legacy GGT1s diverge in only one of the 14 positions.

Although all of the GGT3 are identified by the NCBI CDD conserved domain database (Marchler-Bauer *et al.*, 2011) as GGTs, the GGT3 are highly divergent from the other kingdoms in 11 of the 14 positions (Table 4). Amongst the 48 GGT3 in the 14 positions, there was conservation with the cross kingdom consensus in only 21% of these residues. However, within the GGT3 clade, the amino acids in the 14 positions have been relatively conserved. Of the 14 positions in the 48 GGT3s, 92% had conservation within the GGT3 clade with an additional 3% of the residues that could not be aligned. There was again evidence of greater divergence in duplicated GGTs. In the duplicated Trichoderma-specific GGT3 clade, all three members have the identical 14 position sequence, which differs from the fungal GGT3 sequence in three positions; in the apparent legacy GGT3, each GGT3 has a valine instead of the consensus isoleucine in position 498, and one GGT3 (*Trichoderma virens* 31658) has a second substitution.

At least one GGT3 is present in all 44 Pezizomycotina surveyed and there is conservation of apparently critical amino acids within the clade. Three of the 44 (7%) of the Pezizomycotina had a duplication in GGT3. GGT3 is also present in some but not all Saccharomycotina, including in some members of the whole genome duplication and in the CTG clades, which translate CTG as serine instead of leucine. Consequently, we postulate that GGT1 has been lost in some Saccharomycotina lineages. *Debaromyces hansenni* in the Saccharomycotina duplicated its GGT3.

Our analysis of the GGTs is consistent with ascomycete phylogeny. Tthe Pezizomycotina are monophyletic, and the Sodariomycetes and Leotiomycetes are sister classes (Medina *et al.*, 2011). The Pezizomycotina-specific clade with a GGT1 sub-clade and a Sodariomycete/Leotiomycete-specific GGT2 sub-clade is consistent with a duplication in a Sodariomycete/Leotiomycete ancestor giving rise to the GGT2s. The duplication predated speciation in the Sodariomycetes, includ-

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                                                    1         10        20        30
C.graminicola_GGT1  .............................................MKLSLTSAALVQLLFGIAQPSLAAPSQSTI
C.graminicola_GGT2  .............................................MKPYTPSIWPCYASLLFLAELRPISAATVPQFV
C.graminicola_GGT3  ......................................................MPLTTESLHSAQAGDFARYASRRSVVH.
S.cerevisiae_ECM38  MLLCNRKVPKTLNTCFILHIFTLLTLGVLVSGMPSKMVSFASQETLQRINNLLRGSANRDVDIIAEYLKKDDDDDGGDKD
Sc.pombe_GGT1       MGINTSSAQSSGAASIARSSVNVKSGNRHLSSNKKSATSALEERASRPSILVTFLVLAGTILSLYIWPILSPDLFFANQR
S.pombe_GGT2        .......MSPTDTTPLLYSWDDQSRHQDPDWHKLRNYHGAWYRRISRRRFSQFIFAFGLMTLFVLVYSISSNLHTPTQF
Human_GGT1          .............................................MKKKLVVLGLLAVVLVLVIVGLCLWLPSASKEPDN
Rat_GGT1            .............................................MKNRFLVLGLVAVVLVFVIIGLCIWLPTTSGKPD.
Arabidopsis_GGT1    ..............................................MSLVRTVTIVLFIIAFLQNAAAQKRQ
Arabidopsis_GGT2    .............................................MNSFMSLVRTATIALLLIAFLQNANAVKNL
Arabidopsis_GGT4    .......MRDAIIADPLLAIDHETVAEKKKQSKNLKISLLLLLILLATSGYYSFSDNITTVFLSRQAIDDDHSLSLGTIS
E.coli_GGT          .............................................MIKPTFLRRVAIAALLSGSCFSAAAAPPAPPVSYGVEEDV


                                          40        50        60        70        80
C.graminicola_GGT1  ...............P...KLGAVASESSVCSRIGTDLLEE...GGNAADATVGTVLCVGVIGMYHSGIGGGGF
C.graminicola_GGT2  FN.............PHSGGLGAVASEAVECSKIGRDLIAQ...GGNAVDALVGTTFCVGTVGMYHSGIGGGGF
C.graminicola_GGT3  .S..............TKGIVSCSQPLAAKCGIEVLRA...GGNAADAAVAVAAGLNITEPCSTGIGGDMF
S.cerevisiae_ECM38  HHNIDIDPLPRRPSLTPDRQLLKVGLHGAISSDLEVCSNLTINEVLLKPPGSNAADAAVTQALCKGMVNFFNSGIGGGGY
Sc.pombe_GGT1       CS.......PKYKNKGSQRVVVEGKNGVVATEEETCSQIGVGILKA...GGNAVDAAIASGICIGAVNSFSSGIGGGGF
S.pombe_GGT2        TG.........HKVR........GRRGAVASEVPVCSDIGVSMLAD...GGNAVDAAIASTFCIGVVNFFSSGIGGGGF
Human_GGT1          ............HVYTRAAVAADAKQCSKIGRDALRD...GGSAVDAAIAALLCVGLMNAHSMGIGGGLF
Rat_GGT1            ............HVYSRAAVATDAKRCSEIGRDMLQE...GGSVVDAAIAILCMGLINAHSMGIGGGLF
Arabidopsis_GGT1    QS.........IVKSRGAVATDDGRCSVIGMRVLRE...GGNAIDASVAAALCLGVVSPASSGIGGGAF
Arabidopsis_GGT2    QS.........IVAYHGAVATDDGRCSAIGTNVLRQ...GGNAIDASVAAALCLGVVSPASSGIGGGGF
Arabidopsis_GGT4    DV.........VESENGVVAADDARCSEIGASVLRS...GGHAVDAAVAITLCVGVVNPMSSGIGGGSF
E.coli_GGT          FH.........PVRAKQGMVASVDATATCVGVDILKE...GGNAVDAAVAVGYALAVTHPQAGNLGGGGF


                           90        100       110       120       130       140       150
C.graminicola_GGT1  MLIRSGN.GTYEFIDFRETAPAAAFEDMYRNN.......KSASIYGGLASGVPGELRGLEHLHKKYG..KLPWATVVAP
C.graminicola_GGT2  VVIRDAD.GNYESVDFRESAPAAAFQDMYKGN.......VEGSVRGGLAVGVPSEVRGLEYIHNKYG..SLPWKTVVTP
C.graminicola_GGT3  ILFYDAATGKVSAMNGSGRSAKRATLEAVRKDLGIADGASGKIPMKSVHAVTVPGAAAGWIDTIERFGSGKVSVEQVLAP
S.cerevisiae_ECM38  VVFSGKDDEDHLSIDFREKAPMDSHKFMFENCSLC......SKIGGLAVGVPGELMGLYRLFKERGSGQVDWRDLIEP
Sc.pombe_GGT1       MLIRHPN.GTAHSLNFRETAPAGASKNMFHGNSTL......SQVGGLSVAVPGEIAGYERAWKMYG..SLPWHKLFEP
S.pombe_GGT2        MLIKHPN.ETAQSLTFREIAPGNVSKHMFDKNPML......AQVGPLSIAIPGELAGLYEAWKSHG..LLDWSKLLEP
Human_GGT1          LTIYNSTTRKAEVINAREVAPRLAFATMFN.SSEQ......SQKGGLSVAVPGEIRGYELAHQRHG..RLPWARLFQP
Rat_GGT1            FTIYNSTTRKAEVINAREMAPRLANTSMFN.NSKD......SEEGGLSVAVPGEIRGYELAHQRHG..RLPWARLFQP
Arabidopsis_GGT1    TVVKIAG.GKEIAYDSRETAPLRATENMYGNVDL......KKKGALSVGVPGEVAGLFTAWKQHG..KLPWKRLVTP
Arabidopsis_GGT2    TMIKLAN.GTEVAYDSRETAPLSATEDMYGDNPER......KKKGSLSVGVPGEVAGLYTAWTQHG..KLPWKQLVEP
Arabidopsis_GGT4    LIVSSQKDSKAEAFDMREMAPLAASKDMYKNDASA......KSLGALSMGVPGEIAGLYEAWKRYG..RLPWKPLFEP
E.coli_GGT          MLIRSKN.GNTTAIDFREMAPAKATRDMFLDDQGNPD...SKKSLTSHLASGTPGTVAGFSLALDKYG..TMPLNKVVQP
                                   ▲▲
                                   B


                    160       170       180       190       200       210       220
C.graminicola_GGT1  AVEVARNGFRVTEDLVRYM..DSVTPNG.NFLTEDPSWAVDFAPNGD.RVKLNETMTRKRYADTLDVIAREG.ADAFYT.
C.graminicola_GGT2  AAQLATAGFIVSKDLVRYM..DSAIKDQKNFLVEDPVWAEEFAPNGT.LLQLGDTIYRKRYGATLAKIAEEG.PDVFYA.
C.graminicola_GGT3  AIELGEKGFPVSELTSHSWNSAERQIREASPNFSEMLKKDPKAADGVRAPNPGEIMKNPTLAQTFRALAKDG.KKGFYT.
S.cerevisiae_ECM38  VAKLGSVGWQIGEALG......ATLELYEDVFLTLKEDWSFVLNSTHDGVLKEGDWIKRPALSNMLMELAKNGSVAPFYDP
Sc.pombe_GGT1       TIRLMRDGMPMPKELA......SRIRRPEFSYFKTHPDWSKIFAPEGV.FLHVGEKFYRPALASTLEEIAKFG.PEVFYT.
S.pombe_GGT2        NVKLAREGFPVTRAME......RVLKLPEMAHLLKDPIWQPILMPNGK.VLRAGDKMFRPAYAKTLEIIANKG.IEPFYR.
Human_GGT1          SIQLARQGFPVGKGLA.....AALE.NKRTVIEQQPVLCEVFCRDRK.VLREGERLTLPQLADTYETLAIEG.AQAFYN.
Rat_GGT1            SIQLARHGFPVGKGLA.....RALD.KKRDIIEKTPALCEVFCRQGK.VLQEGETVTMPKLADTLQILAQEG.ARAFYN.
Arabidopsis_GGT1    AEKLAEG.FKISKYLY.....MQMN.ATRSDILADKGLSDLFVSNGE.LKKPGTICHNPKLALTLKLIGEYG.PKAFYN.
Arabidopsis_GGT2    AEKLAAEGFKISKYLY.....MQMN.ATRSDILADKGLSDLFVSNGE.LKKPGAICRNPKLALTLSQIAEYG.PKAFYN.
Arabidopsis_GGT4    AIKLARDGFVVYPYLG.....KAIS.TKVAMILKDPGMRSVFSRNGQ.VLKTGETCYNPELAQSLETISEQG.PGAFYN.
E.coli_GGT          AFKLARDGFIVNDALA.....DDLKTYGSEVLPNHENSKAIFWKEGE.PLKKGDTLVQANLAKSLEMIAENG.PDEFYK.


                    230       240       250       260                         270       280
C.graminicola_GGT1  .GSLANATIAALQRSNGIMTLEDLRNYTVAVREPAQISYRG......................YKLTACNAPS.GGVVALS
C.graminicola_GGT2  .GPIAESIVATARATNGTLTLDDLASYKVVTRPSLSTSYRG...........................HRLVSIGAPA.SGAVCLN
C.graminicola_GGT3  .GRVAEELVKVVKDLGGRLELDDLAYHLEKGSEPVDPISLKFPKPTLKSQANGHGAETNGDDGAVEVWEHPPNGQGIVALM
S.cerevisiae_ECM38  DHWIAKSMIDTVAKYNGIMNLQDVSSYDVHVTKPLSMKIRK......GA.NPIPDNDMTVLTSSGSSSGAALLA
Sc.pombe_GGT1       .GKLAERLVKFVQQQGGILTMEDMANFSVVVEEPIYGNFYD.............................REVITCGSPCSGEALIL
S.pombe_GGT2        .GELTNSMVKFIQDNGGIVTVEDFGNYSTVFADALHTSYRG...........................HDVYTCTLPTSGPALIE
Human_GGT1          .GSLTAQIVKDIQAAGGIVTAEDLNNYRAELIEHPLNISLG..............................DVVLYMPSAPLSGPVLAL
Rat_GGT1            .GSLTAQIVKDIQEAGGIMTVEDLNNYRAEVIEHPMSIGLG..............................DSTLYVPSAPLSGPVLIL
Arabidopsis_GGT1    .GTVGVNLARDIKKSGGIITLKDLQSYRVKIKEPLSADILG..............................YRVLGMPPPSSGGAAMML
Arabidopsis_GGT2    .GTVGPNLVSDIQKAGGIITLKDLQNYNVKVKEPLSTEILG..............................YRLLGMPPPSSGGPAMML
Arabidopsis_GGT4    .GTVGEKLVKDVKKAGGIIMDDLRSYKVRVTDAMSVDVMG..............................YTVHGMPPPSSGGTVGFSM
E.coli_GGT          .GTIAEQIAQEMQKNGGLITKEDLAAYKAVERTPISGDYRG......................YQVYSMPPPSSGGIHIVG


                    290       300       310       320       330       340       350
C.graminicola_GGT1  ALNIVSGYEGFGD.....P..AARNQTVHRIDCAMRFAYGQR.TKLGDPSFVDG.LAAYTKDMLSPEVGAEIRSKISDVR
C.graminicola_GGT2  TLKIMEQFDPAESA.......DAKLG.VHRFDFALRFAYGAR.TLLGDPDFVDG.IGPFEDTLLDEDTARDIRRRILDNQ
C.graminicola_GGT3  ALGIIQELQASGVVSAWSPEDHNTAPYLHAIIEALRLGFTDASWVYTDPNVVPVPSAALISREYSAARAKLFDPNRATQT
S.cerevisiae_ECM38  ALRIMDNFQNQEGG.......DYEKETTYHLLESMKWMASAR.SRLG..DFEGEALPKHIEEVLDPEWALKAVKSIKRNS
Sc.pombe_GGT1       GLNVLSKVDLSEGTSILGC..EMTDIGVHHLIETMKWMSAGR.TVLADPTFYN..NTDHVEQLLSLEYADEIRNNISNER
S.pombe_GGT2        GLNILDGYPLNTPS.......LAFPKRLHLEVEAMKWLSAGR.TQFGDPDFLPLDHLDVVSKLLSKEFASQIRNNISLSK
Human_GGT1          ILNILKGYNFSRES.VESP..EQKGLTYHRIVEAPRFAYAKR.TLLGDPKFVD..VTEVVRNMTSEFFAAQLRAQISDDT
Rat_GGT1            ILNILKGYNFSPKS.VATP..EQKGLTYHRIVEAPRFAYAKR.TMLGDPKFVD..VSQVIRNMSSEFYATQLRARITDET
Arabidopsis_GGT1    VLNILSQYGIPSGVSG......PLGVHRLIEALKHAFAVR.MNLGDPDFTD..VTKVVSDMLSPKFAKDLKSKINDQK
Arabidopsis_GGT2    ILNILAQYGIPSGVSG......PLGVHRLIEALKHAFAVR.MNLGDPDFVPE.VTNVVADMLSPKFAQDLKSKINDEK
Arabidopsis_GGT4    VMNILDSYGIPSNLYTASGR.......ELGLHRLIEAMKHHMFAAR.MDLGDPEFVN..VTNSMNQMLSKAHAEEIQKRIFDNT
E.coli_GGT          ILNILENFDMKKYG.......FGSADAMQIMAEAEKYAYADRSEYLGDPDFVK..VP..WQALTNKAYAKSIADQIDINK
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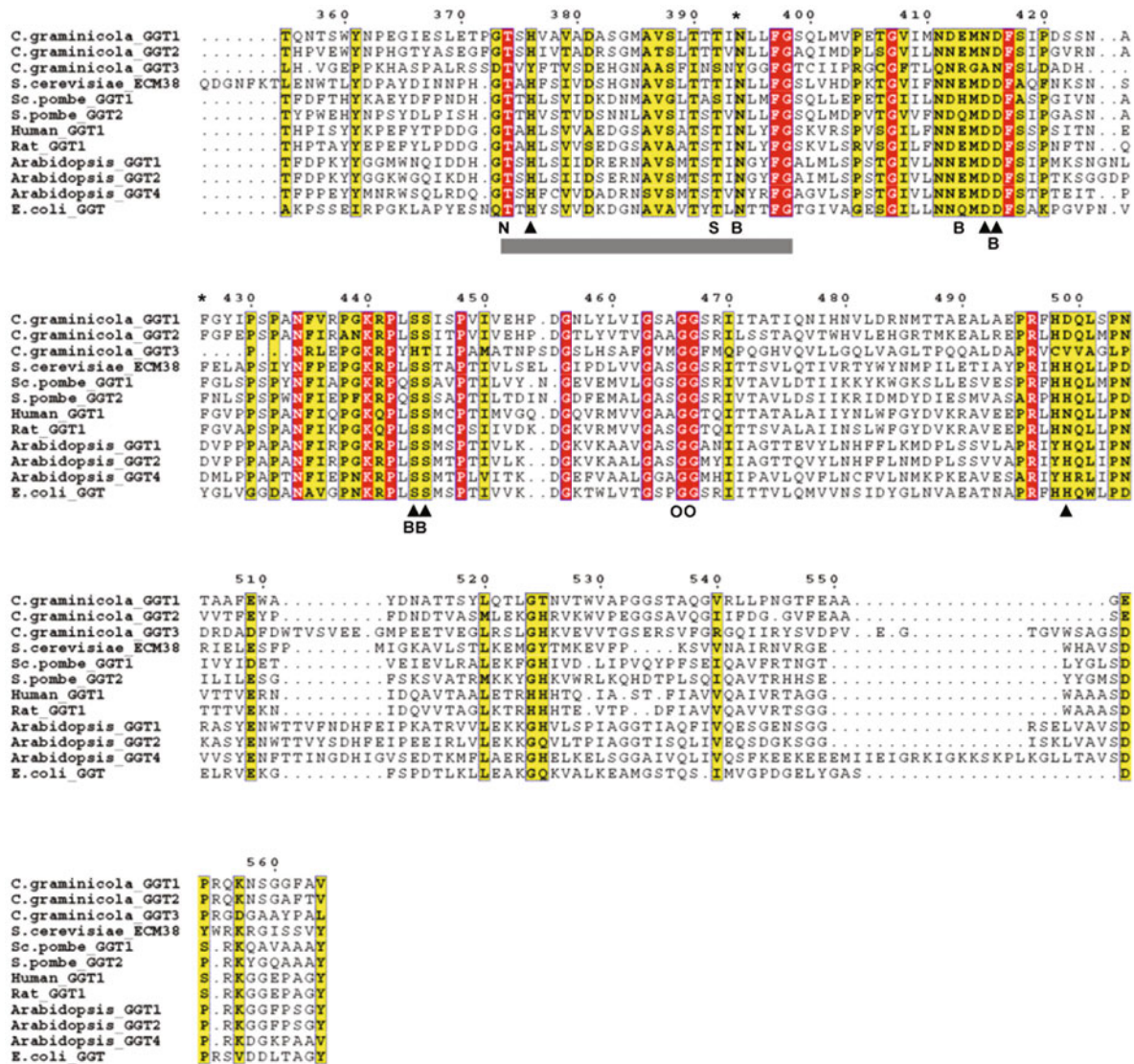
Fig. 3. Continued

Fig. 3. Multiple sequence alignment of the three γ-glutamyl transferases (GGT) in *C. graminicola* and characterized GGTs (with GenBank accession nos.) from *S. cerevisiae* ECM38 (NP_013402.1), *S. pombe* (GGT1, NP_593457.1; GGT2, NP_593273), human GGT1 (P19440.2), rat GGT1 (P07314.4), *A. thaliana* (GGT1, AT4G39640.1; GGT2, AT4G39650.1; GGT4, AT4G29210.1) and *E. coli* K12 (BAE77846.1). Residues that are identical in all sequences are boxed in red, and similar residues are filled with yellow. GGT residues in human GGT that had 66 to 100% less enzyme activity after site-directed mutagenesis (R107K, R107Q, R107H, E108Q, H383A, D422A, D423A, S451A, S452A, H505A with residue numbers for the human GGT1) are marked with solid triangles (Ikeda *et al.*, 1993, 1995a, 1995b, 1996). Letters N, S, B, O, and the asterisk correspond to the residues for the catalytic nucleophile, stabilizing the nucleophile, substrate binding, the oxyanion hole, and the substrate-binding pocket wall in *E. coli*, respectively (Okada *et al.*, 2006). The GGT signature motif T-[STA]-H-x-[ST]-[LIVMA]-x(4)-G-[SN]-x-V-[STA]-x-T-x-T-[LIVM]-[NE]-x(1,2)-[FY]-G (PROSITE PS00462) is shown above the gray bar.

ing in the two examined *Colletotrichum* spp., four fusaria (three *Fusarium* spp. and *Nectria haematococca*) and two *Verticillium* spp. Interestingly, the *C. graminicola* CgGGT1 which is in the GGT1 clade, and CgGGT2, which is in the GGT2 clade, are located on the same chromosome. The Saccharomycotina are also a monophyletic group and the Saccharomycotina and the Pezizomycotina are generally considered to be sister taxa (Medina *et al.*, 2011), consistent with the composition of GGT3, which does not include Taphrinomycotina. There is conflicting evidence of whether the Taphrinomycotina are a separate lineage (Medina *et al.*, 2011) or whether the Saccharomycotina and the Taphrinomycotina are sister taxa (Robbertse *et al.*, 2006), perhaps con-

sistent with the Saccharomycotina/Taphrinomycotina clade. Regardless, the Saccharomycotina and the Taphrinomycotina GGTs are in distinct sub-clades, and the conservation of critical amino acids in this clade is consistent with an ancient origin of these GGTs.

In addition to evolutionary conservation and some duplication of GGTs, there have been loses of GGTs. For example, the ancestral *Neurospora* sp. in the Sordariomycetes appears to have lost its GGT1, since none of the three examined *Neurospora* species have a copy of GGT1.

Of the 44 surveyed Pezizomycotina, the Pezizomycotina have a mode of two GGTs, a mean of 2.6 and a range of two to four. This study is the first examination of the evolution

Table 2. Conservation and limited diversity in the Saccharomycotina/Taphrinomycotina γ-glutamyltransferase (GGT) clade in the cross-kingdom highly conserved amino acid positions

	Cross-kingdom conservation of critical amino acids[a]													
	99	100	374	376	392	394	413	415	416	444	445	466	467	498
	R	E	T	H	T	N	E,Q	D	D	S	S	G	G	H
Accessions from the selected species in the Saccharomycotina/Taphrinomycotina clade	Amino acids in the Saccharomycotina/Taphrinomycotina GGT clade[b]													
Ashbya gossypii ERGO0A07964, *Candida albicans* CAWG 01318.1, *C. glabrata* CAGL0I00506p, *Debaromyces hansenni* DEHA0D10065p, *Kluveromyces lactis* KLLA0C05236p, *Saccharomyces cerevisiae* ECM38/YLR299W, *S. kluyveri* SAKL0A 07634p, *Schizosaccharomyces japonicus* SJAG_06139.4, *Yarrowia lypolitica* YALI0C 11363p	**R**	**E**	**T**	**H**	**T**	**N**	**E**	**D**	**D**	**S**	**S**	**G**	**G**	**H**
Schizosaccharomyces octosporus SOCG_02239.5, *S. pombe* SPAC56E4.06c	-	-	-	-	-	-	Q	-	-	-	-	-	-	-
Schizosaccharomyces japonicus SJAG_01612.4[c]	-	-	-	-	-	-	H	-	-	-	-	-	-	-
Schizosaccharomyces octosporus SOCG_01537.5[c], *S. pombe* SPAC664.09[c]	-	-	-	-	S	-	H	-	-	-	-	-	-	-
Candida guillermondi PGUG 04722.1	-	-	-	-	-	-	Q	?	?	?	?	?	?	?

[a] The consensus sequence (with GenBank accession nos.) shown in Fig. 3 includes the human GGT1 (P19440.2), rat GGT1 (P07314.4), *A. thaliana* (GGT1, AT4G39640.1; GGT2, AT4G39650.1; GGT4, AT4G29210.1) and *E. coli* K12 (BAE77846.1). Residues essential for GGT activity in human and *E. coli* GGTs (Ikeda *et al.*, 1993, 1995a, 1995b, 1996; Okada *et al.*, 2006; prosite.expasy.org/PDOC00404) were selected. Amino acid positions are shown in Fig. 3. Positions that were identified as critical in both human and *E. coli* are darkly shaded. Positions in human GGT that had 66 to 100% less enzyme activity after site-directed mutagenesis studies are lightly shaded. Positions identified by crystallography that are critical for catalysis in the *E. coli* GGT but not in the human GGT are not shaded.
[b] The consensus sequence for the fungal clade is shown in bold. Divergence from the cross-kingdom consensus is indicated by yellow fill. Divergence from the fungal clade is indicated by italics. -, same residue as in the Saccharomycotina/Taphrinomycotina-specific clade consensus; ?, gap in alignment.
[c] A duplicated gene.

Table 3. Conservation and diversity in the cross-kingdom conserved amino acid positions in the γ-glutamyltransferase (GGT) Pezizomycotina-only clade

	Cross-kingdom conservation of critical amino acids[a]													
	99	100	374	376	392	394	413	415	416	444	445	466	467	498
	R	E	T	H	T	N	E,Q	D	D	S	S	G	G	H
Accessions in the selected species in the GGT1 sub-clade	Amino acids in the Pezizomycotina-only clade[b]													
Alternaria brassicicola AB07869.1, *A. oryzae* AO090023000537[c], *Cochliobolus heterostrophus* 88332, *C. sativus* 147404, *Colletotrichum graminicola* GLRG_09590, *C. higginsianum* CH063 11845.1, *Fusarium graminearum* FGSG 09141.3, *F. oxysporum* FOXG 05788.2, *F. verticillioides* FVEG 03657.3, *Leptosphaeria maculans* Lema T102270.1, *Nectria haematococca* 96628, *Paracoccidioides brasiliensis* PADG 07986.1, *Pyrenophora teres* PTTG 17142.1, *Setosphaeria turcica* 166295, *Trichoderma atroviridae* 234843, *T. virens* 213573, *Verticillium albo-atrum* VDBG 07889.1, *V. dahliae* VDAG 05455.1	**R**	**E**	**T**	**H**	**T**	**N**	**E**	**N**	**D**	**S**	**S**	**G**	**G**	**D**
Botrytis cinerea BC1G 07620.1, *Coccidioides immitis* CIHG 09375.1, *Dothistroma septosporum* 75017, *Mycosphaerella fijiensis* 204479[c], *M. graminicola* 45394, *Oidiodendron maius* 147533[c], *Sclerotinia sclerotiorum* SS1G_14127.3	-	-	-	A	-	-	-	-	-	-	-	-	-	-
Pyrenophora tritici-repentis PTRG 02864.1	-	-	-	-	-	-	Q	-	-	-	-	-	-	-
Stagonospora nodorum SNOG 03494	-	-	-	-	-	-	-	-	-	-	-	-	-	?
Aspergillus nidulans ANID 10444.1, *A. oryzae* AO090005000169[c], *Microsporum canis* MCYG 05524.2[c], *M. gypseum* MGYG 02615.2[c], *Trichoderma reesei* 69811, *Trichophyton rubrum* TERG 06701.2[c]	-	-	-	-	-	-	D	-	-	-	-	-	-	-
Aspergillus fumigatus Afu7g04760	-	-	?	?	-	-	D	-	-	-	-	-	-	-
Aspergillus oryzae AO090113000029[c]	-	-	-	-	-	-	-	D	-	-	A	-	S	H
Histoplasma capsulatum HCAG 03238.3	-	-	-	-	-	-	D	D	-	-	-	-	-	-
Magnaporthe grisea MGG 02134.6	-	-	-	-	-	-	N	D	-	-	-	-	-	-
Microsporum canis MCYG 01934.2[c]	-	P	-	-	S	G	V	A	-	-	G	-	-	-
Microsporum gypseum MGYG 04136.2[c]	-	T	-	-	S	G	D	S	-	-	G	-	-	-
Trichophyton rubrum TERG 04463.2[c]	-	T	-	-	S	G	D	S	-	-	G	-	-	-
Accessions in the GGT2 sub-clade														
Colletotrichum graminicola GLRG_05260, *Magnaporthe grisea* MGG 03882.6, *Oidiodendron maius* 183846[c], *Trichoderma atroviridae* 87687, *T. reesei* 80911, *T. virens* 50329, *Verticillium albo-atrum* VDBG 05559.1, *V. dahliae* VDAG 07501.1	**R**	**E**	**T**	**H**	**T**	**N**	**E**	**N**	**D**	**S**	**S**	**G**	**G**	**D**
Colletotrichum higginsianum CH063 11268.1	-	-	-	-	-	-	-	-	-	-	-	-	-	?
Fusarium graminerarum FGSG 10859.3, *F. oxysporum* FGSG 10859.3, *F. verticillioides* FVEG 11538.3, *Nectria haematococca* 93304	-	-	-	-	-	-	-	-	-	-	-	-	-	N
Botrytis cinerea BC1G 12271.1	-	-	-	-	-	-	D	-	-	?	?	-	-	?
Neurospora crassa, *N. discreta* 128264, *N. tetrasperma* 65159, *Sclerotinia sclerotiorum* SS1G_10940.3	-	-	-	-	-	-	D	-	-	-	-	-	-	-
Accessions from the Pezizomycotina-only clade that are neither in the GGT1 nor the GGT2 sub-clades														
Microsporum gypseum MGYG 07028.2[c]	**R**	**E**	**T**	**H**	**T**	**N**	*G*	**D**	**D**	**S**	**S**	**G**	**G**	**D**
Mycosphaerella fijiensis 32768[c]	-	-	-	-	S	-	E	N	-	-	-	-	-	-
Oidiodendron maius 105638[c]	-	-	A	Y	-	-	A	S	-	-	-	-	C	-

[a] See Table 2 footnote (a).
[b] The consensus sequence for the fungal clade is shown in bold. Divergence from the cross-kingdom consensus is indicated by fill. Divergence from the fungal clade is indicated by italics. -, same residue as the sub-clade consensus; ?, gap in alignment
[c] Gene was duplicated.

Table 4. High divergence of the Pezizomycotina-Saccharomycotina GGT3 clade from the typically conserved, cross-kingdom amino acid positions, with relative conservation of amino acids within the GGT3 clade

	Cross-kingdom conservation of critical amino acids[a]													
	99	100	374	376	392	394	413	415	416	444	445	466	467	498
	R	E	T	H	T	N	E,Q	D	D	S	S	G	G	H
Accessions in the selected species in the GGT3 clade	Amino acids in the GGT3 clade[b]													
Alternaria brassicicola AB09738.1, *Aspergillus fumigatus* Afu4g13580, *A. nidulans* ANID 05658.1, *A. oryzae* AO090009000211, *Cercospora zea maydis* 34333, *Cochliobolus heterostrophus* 1137353, *C. sativus* 349745, *Dothistroma septosporum* 51997, *Fusarium graminerarum* FGSG 12231.3, *F. oxysporum* FOXG 13668.2, *F. verticillioides* FVEG 11101.3, *Leptosphaeria maculans* Lema T016160.1, *Magnaporthe grisea* MGG 09541.6, *Microsporum canis* MCYG 01019.2, *M. gypseum* MGYG 00967.2, *Mycosphaerella graminicola* 65045, *Nectria haematococca* 47582, *Neurospora crassa* NCU04130.5, *N. discreta* 18172, *N. tetrasperma* 143849, *Paracoccidioides brasiliensis* PADG 01479.1, *Pyrenophora teres* PTTG 09621.1, *P. tritici-repentis* PTRG 01602.1, *Setosphaeria turcica* 30176, *Stagonospora nodorum* SNOG 06623, *Trichophyton rubrum* TERG 00831.2, *Verticillium albo-atrum* VDBG 02110.1	**S**	**G**	**T**	**Y**	**S**	**Y**	**R**	**A**	**N**	**H**	**T**	**G**	**G**	**I**
Botrytis cinerea BC1G 07070.1	-	-	-	-	-	F	-	G	-	-	-	-	-	-
Candida albicans CAWG 03454.1	-	-	-	-	-	-	-	-	N	-	-	-	-	L
Candida guillermondi PGUG 04562.1	C	-	-	-	-	-	-	-	-	-	-	-	-	L
Coccidioides immitis CIHG 05352.1	-	-	-	-	-	-	-	-	S	-	-	-	-	?
Colletotrichum graminicola GLRG_04161, *Trichoderma atroviridae* 296510, *T. reesei* 5645	-	-	-	-	-	-	-	-	-	-	-	-	-	V
Colletotrichum higginsianum CH063 09808.1	-	-	P	P	?	?	-	-	-	-	-	-	-	-
Debaromyces hansenni DEHA0C17160p	T	-	-	-	-	-	-	G	-	-	-	-	-	L
Debaromyces hansenni DEHA0G18689p, *Saccharomyces kluyveri* SAKL0D07854p	T	-	-	-	-	-	-	-	-	-	-	-	-	L
Histoplasma capsulatum HCAG 06177.3	-	-	-	-	-	?	?	?	?	?	?	?	?	?
Kluveromyces lactis KLLA0F23012p	T	-	-	-	-	-	-	-	-	-	-	-	-	L
Oidiodendron maius 190747	-	-	-	-	-	F	-	G	-	-	-	-	-	-
Sclerotinia sclerotiorum SS1G_05330.3	?	?	-	-	-	F	-	G	-	-	-	-	-	-
Trichoderma atroviridae 77856[c], *Trichoderma reesei* 5337[c], *T. virens* 64824[c]	-	-	-	-	-	-	A	-	T	Q	-	-	-	-
Trichoderma virens 31658	-	-	-	-	-	-	-	-	G	-	-	-	-	V
Verticillium dahliae VDAG 02575.1	-	-	-	-	-	?	?	?	?	?	-	-	-	-
Yarrowia lypolitica YALI0C20669p	-	-	-	-	-	-	-	-	-	-	-	-	-	L

[a] See Table 2 footnote (a).
[b] The consensus sequence for the fungal clade is shown in bold. Divergence from the cross-kingdom consensus is indicated by fill. Divergence from the fungal clade is indicated by italics. -, identical residue as the GGT3 consensus clade; ?, gap in alignment.
[c] Duplicated clade.

of fungal GGTs and consequently the first recognition of the three major ascomycete GGT clades. The GGTs in the two Pezizomycotina-containing main clades are divergent from the relatively well-studied members of the Saccharomycotina/Taphrinomycotina-only clade. *Ss-Ggt1* is a member of the GGT1 clade. We are unaware of any mutational analyses of a member of either GGT 2 or GGT3, and the enzyme activity of neither a GGT2 nor a GGT3 has been confirmed.

Expression of CgGGT1 in *P. pastoris* and demonstration that CgGGT1 is a γ-glutamyltransferase (EC 2.3.2.2)

To date, the only fungal GGTs in which GGT activity has been demonstrated are in the yeasts *S. cerevisiae* in the Saccharomycotina and in *S. pombe* and *Hansenula polymorpha* in the Taphrinomycotina (Mehdi and Penninckx, 1997; Park *et al.*, 2004, 2005; Ubiyvovk *et al.*, 2006). Of the three *Colletotrichum graminicola* GGTs, *CgGGT1* mRNA (from gene GLRG_09590) is the most highly expressed (Bello and Epstein, unpublished). Because the Pezizomycotina GGT1 and GGT2 clades differ in two apparently critical amino acids from the cross-kingdom consensus, *CgGGT1* was cloned and the protein product was expressed as a secreted protein in

Pichia pastoris in order to verify that *CgGGT1* encodes a γ-glutamyltransferase. Western blot analysis, using an anti-*c-myc*-peroxidase mouse monoclonal antibody, indicated that transformants with *CgGGT1* secreted two intensely-labeled recombinant compounds into the media, in contrast to the non-transformed strain (Fig. 4A). In humans and rodents, GGT is heterogeneously glycosylated and separates as a broad band (Harding *et al.*, 1997; West *et al.*, 2011). The expected molecular masses of the recombinant but unglycosylated CgGGT1 are the following from the 5′ end: the α-factor (9.3 kDa) which is generally cleaved; the heavy chain (39.8 kDa), the light chain (20.2 kDa), and the *c-myc*/his tag (2.5 kDa). The upper band has a range of molecular weight from 62 kDa, consistent with an unglycosylated heavy chain + light chain + *c-myc*/his tag, to 119 kDa, with an average of 85 kDa, consistent with the heterogeneous glycosylation and possibly by a retained α-signal. The lower band has a range of molecular weight from 30 kDa, consistent with a heterogeneously glycosylated light chain + *c-myc*/his tag to 41 kDa, with an average of 35 kDa, consistent with the heterogeneous glycosylation. The supernatants of three selected recombinants were then tested for γ-glutamyltransferase

activity (Fig. 4B). For negative controls, cell-free culture supernatant of a strain transformed with pPICZαA without the *CgGGT1* insert and the untransformed strain (KM71H) were used. A one-way ANOVA indicated a highly significant strain effect ($P<0.0001$). The supernatant of the three recombinant strains produced an average of 2.4±0.1 μmol *p*-nitroanilide mg^{-1} soluble cell protein h^{-1}, which is equivalent to an average of 32±1 times the average of the two negative controls. Consequently, CgGGT1 is a *bona fide* γ-glutamyltransferase.

In addition to demonstrating that a member of GGT1 has *bona fide* γ-glutamyltransferase activity, this apparently is the first time that a fungal GGT has been expressed in a heterologous system. Consequently, we have demonstrated that *P. pastoris* is a suitable expression system for the production of large quantities of active, eukaryotic GGTs from culture supernatant. Such cell lines will facilitate production, purification and further characterization of GGTs *in vitro*.

Acknowledgements

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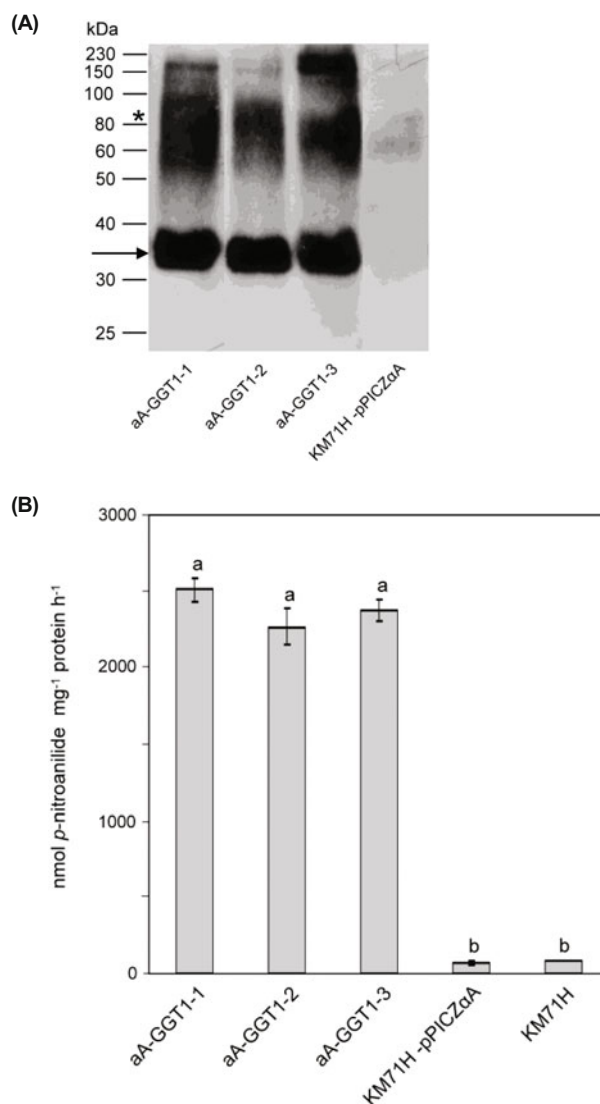

Fig. 4. γ-Glutamyltransferase (GGT) secreted from *P. pastoris* transformed with *C. graminicola CgGGT1* cDNA. After an 8-day methanol induction, cell-free supernatants were collected from three recombinant CgGGT1 colonies, and the wild type KM71H transformed with the empty vector pPICZαA as a negative control. (A) Detection of CgGGT1 recombinant secreted protein by western blot analysis with an anti-*c-myc* antibody. The 85 kDa band, consistent with a glycosylated heavy and light chain fused with the *c-myc*-his tag, is shown with an asterisk. The 35 kDa band, consistent with a glycosylated light chain fused with the *c-myc*-his tag, is shown with an arrow. (B) GGT activity was measured using γ-glutamyl *p*-nitroanilide as donor and glycylglycine as acceptor substrate. GGT activity in the cell-free supernatant is expressed per mg of soluble protein in the *P. pastoris* cells that produced the supernatant. Means±SE of three biological replicates are shown. The untransformed wild type KM71H was also included as a second negative control.

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