Expression of *Candida albicans* glutathione transferases is induced inside phagocytes and upon diverse environmental stresses

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Abstract

Candida albicans has four open reading frames for glutathione transferases of the GTT classes, and another one coding for an Omega class member. In laboratory conditions, only *GTT11* (GTT1/2 class) and *GTO1* (Omega class) are expressed significantly in exponentially-growing cells, particularly when these are subjected to diverse environmental stresses, including oxidative stress. They also become transitorily upregulated at early stationary phase. Accordingly, the levels of the CaGto1 and CaGtt11 proteins increase after treatment with oxidants and upon osmotic stress, in addition to early stationary phase. *GTT11* and *GTO1* transcription shows a complex dependence on the Hog1 and Cap1 factors upon different stresses. Purified CaGtt11 and CaGto1 proteins display enzyme activities similar to the *Saccharomyces cerevisiae* homologues. Thus, CaGtt11 has activity against standard glutathione transferase substrates and is also active as peroxidase, while CaGto1 displays thiol oxidoreductase and dehydroascorbate reductase activities. Fluorescence microscopy and subfractionation studies indicate that CaGto1 is cytosolic, while CaGtt11 is associated to a particulate fraction. In *ex vivo* conditions, CaGto1 and CaGtt11 become transitorily upregulated inside macrophages and neutrophils. In these conditions the promoter of *GTT14* (GTT1/2 class) also becomes activated. These observations point to the importance to *C. albicans* glutathione transferases in the defence against phagocytes.

Introduction

Glutathione transferases (GSTs) form a superfamily of enzymes which conjugate xenobiotics or their metabolites to glutathione (GSH), followed by elimination of the conjugates or their internalization in cell compartments such as vacuoles (Hayes et al., 2005; Frova, 2006). Thus, they mediate detoxification of a large variety of organic compounds. Most GSTs share a common structure composed by an N-terminal domain with a thioredoxin-fold structure that includes the active site for the nucleophilic attack on substrates, followed by a α-helix-rich region with the substrate recognition site. GSTs have been classified into three main subfamilies based on their structure and cellular location: cytosolic, microsomal and mitochondrial (Kappa class) GSTs. Cytosolic GSTs have been the more studied ones, particularly those of animal cells, although they also exist in other taxa (Frova, 2006). Given their heterogeneity, cytosolic GSTs have been divided into classes depending on sequence, substrate specificity, or immunological properties. Some classes (such as Zeta, Theta, Omega or Sigma) are common to different taxa, while others are more specific. The heterogeneity of the GST superfamily helps to explain why in addition to (or instead of) the detoxification role, many GSTs play roles in cellular functions as diverse as modulation of signalling processes, leukotriene and prostaglandin biosynthesis, amino acids catabolism, or defence against oxidative stress (Hayes et al., 2005; Frova, 2006). GSH-dependent peroxidase or thiol oxidoreductase enzymatic activities have been also demonstrated for some GSTs.

Fungi also contain GSTs, although these are structurally and immunologically different from other organisms (McGoldrick et al., 2005; Morel et al., 2009). Six different classes of fungal GSTs have been proposed on the basis of phylogenetic analyses and sequence comparisons: GTT1, GTT2, Omega, URE2-like, EFBγ and MAK16 (Morel et al., 2009). Most experimental data are derived from studies in *Saccharomyces cerevisiae*, and involve the first four classes. The GTT1 and GTT2 classes are fungal-specific and each one has one member in *S. cerevisiae*, respectively named Gtt1 and Gtt2. Both of them act on standard GST substrates such as 1-chloro-2,4-dinitrobenzene (CDNB). Gtt1 and Gtt2 are functionally related to Grx1 and Grx2 glutaredoxins, providing defence against oxidants in collaboration with the two glutaredoxins (Collinson & Grant, 2003; Mariani et al., 2008). Gtt1 has been shown to be associated with the endoplasmic reticulum (Choi et al., 1998). The *Schizosaccharomyces pombe* homologues of Gtt1 and Gtt2 also confer defence against hydroperoxides (Veal et al., 2002). This role against oxidative stress could be related to the described glutathione peroxidase activity of *S. cerevisiae* Gtt1 (Garcerá et al., 2006).

Omega-class GSTs are present in many different organisms and are characterized by their low or null activity against standard GST substrates. On the contrary, they are active as glutaredoxins/thiol oxidoreductases and dehydroascorbate reductases (DHAR) through a single cysteine residue at the active site (Whitbread et al., 2005). *S. cerevisiae* contains three Omega-class GSTs, named Gto1, Gto2 and Gto3 (Garcerá et al., 2006). Gto1 is peroxisomal and is involved in sulphur amino acid metabolism, while Gto2 and Gto3 are cytosolic and their functions have not been discerned (Barreto et al., 2006). The absence of Gto1, Gtt1 and Gtt2 together causes hypersensitivity to cadmium in *S. cerevisiae*, probably due to the GSH depletion observed in the triple mutant (Barreto et al., 2006). In fact, GSTs protect against cadmium toxicity in yeast cells by mediating sequestration of cadmium-glutathione complexes at the vacuolar compartment (Li et al, 1997; Adamis et al., 2004). No phenotypes related to oxidative stress have been observed for *S. cerevisiae* mutants lacking one or both *GTO2* and *GTO3* genes, although expression of these two genes, particularly of *GTO2*, is upregulated upon the action of diverse oxidants (Barreto et al., 2006). An antioxidant role has been demonstrated for the Omega GST *GSTO-1* in transgenic variants of *Caenorhabditis elegans* (Burmeister et al., 2008). The genetic association between human Omega GSTs and the age of onset of Alzheimer's and Parkinson's diseases also points to the relationship between these GSTs and oxidative stress protection (Li et al., 2003). In addition to their thiol oxidoreductase activity, recent studies have attributed other enzyme activities to Omega-class GSTs. Thus, they participate in the transformation of inorganic arsenic to dimethylarsinate (Schmuck et al., 2005), which may explain the upregulation of an Omega GST in rice roots during arsenic stress (Ahsan et al., 2008). *Drosophila melanogaster* has an Omega GST with pyrimidodiazepine synthase activity, which catalyzes a key step in the synthesis of the eye pigment drosopterin (Kim et al., 2006).

The *S. cerevisiae* Ure2 protein is a regulator of nitrogen catabolic gene expression which exhibits structural characteristics of GSTs, although it lacks GST activity on substrates such as CDNB (Choi et al., 1998). However, it has glutathione peroxidase (Bai et al., 2004) and thiol oxidoreductase (Zhang & Perrett, 2009) activities, and confers resistance to heavy metals when growing with ammonia as the only nitrogen source (Rai & Cooper, 2005). The Ure2 protein from *S. cerevisiae* has prion-like properties, but most of the fungal sequences with homology to Ure2 lack the protein region conferring such prion properties (Morel et al., 2009).

Candida albicans is a pathogenic fungus which has to cope with oxidative conditions during host infection, particularly inside phagocytic cells. Based on the relationships between some fungal GSTs and oxidative stress, in this study we have

analyzed the *Candida albicans* members of the GTT (with standard GSH-conjugating activity) and the omega classes (with thiol oxidoreductase activity) and their response when cells are exposed to oxidants.

Materials and Methods

Strains, plasmids and genetic manipulations

The *C. albicans* strains employed in this work are described in Table 1. Recombinant proteins were expressed in *Escherichia coli* BL21 cells (Novagen). For this purpose, plasmid pMM801 was constructed, containing the *GTT11* open reading frame (ORF) cloned between the *Nde*I and *Xho*I sites of the expression vector pET-21a (Novagen). Similarly, pMM803 contains the *GTO1* ORF cloned between the *Nhe*I and *Xho*I sites of pET-21a. In both cases, adequate oligonucleotides were employed for PCR amplification of the ORF without the initial and stops codons, from genomic DNA of the CAI-4 strain. The *C. albicans* codon-optimised GFP sequence from pGFP (Barelle et al., 2004) was PCR-amplified and cloned between the *EcoR*V and *Mlu*I sites of CIp10, which is a *C. albicans* integrative plasmid with the *CaURA3* marker (Murad et al., 2000). The resulting plasmid was named pMM812. This plasmid was then used to clone 1000 bp of the *GTO1* promoter (from position -1 relative to the start codon) followed by the entire coding sequence in frame with the GFP sequence at the C-terminus, to create plasmid pMM814. Similarly, pMM833 derives from pMM812 and contains 820 bp of the *GTT11* promoter, plus the gene coding sequence in frame with the GFP sequence. Plasmids pMM814 and pMM833 were integrated at the chromosomal *RP10* locus of CAI-4 by homology recombination after plasmid linearization (Murad et al., 2000), resulting in strains MML968 and MML970 respectively (Table 1). Plasmid pMM843 contains 807 bp of the *GTT14* promoter, beginning at position -1, cloned between the *Xho*I and *Hind*III sites of pGFP for expression of the GFP protein. *C. albicans* MML995 contains a linearized copy of pMM843 integrated at the CAI-4 *RP10* locus. Similarly, strain MML972 contains a chromosomally-integrated copy of the pGFP vector in the *RP10* locus of CAI-4. All plasmid constructions were checked by DNA sequencing.

Growth conditions

C. albicans cells were usually grown at 30ºC in YPD medium (2% glucose, 2% peptone and 1% yeast extract, plus 2% agar if required). In some cases, synthetic SC medium (Sherman 2002) was employed. For selection of transformants, the auxotrophic

requirement corresponding to the plasmid selection marker was omitted. Growth in liquid medium was done in aerated conditions. For induction of cell filamentation, 10% fetal bovine serum (Invitrogen) was added to exponentially growing cells in YPD medium, and growth was continued at 37ºC.

Purification of recombinant proteins

E. coli cultures transformed with the corresponding plasmid derived from pET-21a were grown in LB medium plus ampicillin (100 μg/ml) at 37ºC, and induced with IPTG as described in Garcerá et al. (2006). Recombinant proteins were purified from cell extracts by affinity chromatography using Ni-NTA-agarose columns, following the manufacturer's instructions. Protein purity was tested by SDS-acrylamide gel electrophoresis followed by Coomassie blue staining.

Determination of enzyme activities

GST activity was spectrophotometrically determined by measuring the conjugation of GSH to CDNB (Habig et al., 1974). Thiol oxidoreductase (glutaredoxin) activity was determined using β-hydroxyethyl disulphide (HED) as substrate, through the reduction of the mixed disulfide formed between HED and GSH (Holmgren & Aslund, 1995). DHAR activity was measured as described in Whitbread et al. (2005). Peroxidase activity was assayed as described previously (Collinson et al., 2002).

Northern and Western blot analyses

Electrophoresis of RNA, probe labelling with digoxigenin, hybridization, and signal detection were done as previously described (Garí et al., 1997). Signals were quantified with the Lumi-Imager equipment (Roche Diagnostics) software. Gene probes were generated from genomic DNA by PCR, using oligonucleotides designed to amplify internal ORF regions. Western blot analyses were done as in Bellí et al. (1998), using anti-GFP polyclonal antibodies (1:500 dilution).

Subcellular fractionation

Yeast cells were grown overnight in YPD medium at 30ºC to 2 x 10^7 cells/ml. About 10^9 cells were collected by centrifugation (4200 g, 5 min) at 4ºC, and washed once with sterile water. For converting cells into spheroplasts, first they were resuspended in 1 ml of reducing buffer [50 mM potassium phosphate (pH 7.4), 0.5 mM EDTA, 50 mM DTT and pronase H (5 mg/ml)] and incubated at 30ºC for 30 min. Next, the cells were spun, resuspended in spheroplasting buffer (KCl 0.6 M) plus Zymolyase 20T [15 mg per g of cells (dry weight)] and incubated at 30ºC until sph eroplast formation was almost

complete (usually about 30-60 min). Spheroplasts were collected by centrifugation (2500 g, 5 min) at 4℃, and then they were broken b y brief sonication. After removal of the intact cells by centrifugation (180 g, 5 min) at 4℃, the total lysate was fractionated by low-speed centrifugation (13000 g, 10 min) at 4 ℃, resulting in supernatant (S13) and pellet (P13) fractions. The S13 fraction was further fractionated by high-speed centrifugation (100000 g, 30 min) at 4℃, to obtain supernatant (S100) and pellet (P100) fractions.

Determination of protein expression in phagocytic cells

The RAW 264.7 mouse macrophage cell line was employed, basically as described in Arana et al. (2007). Cells were grown in RPMI 1640 medium supplemented with 10 % heat-inactivated fetal bovine serum, glutamine and 1% streptomycin/penicillin at 37ºC in 5% CO_2. About 5 x 10^5 cells were seeded the day before the experiment in 35 mm plates containing RPMI 1640 medium. *C. albicans* control cells (MML972), or derivatives expressing CaGto1-GFP (MML968) or CaGtt11-GFP (MML970) that were growing exponentially in YPD medium, were collected, opsonized with 50% human AB serum (Sigma), washed, resuspended in RPMI 1640 medium and added to macrophage cultures at a ratio between 1:20 and 1:40 (yeast: macrophages). After infection, the plates were returned to the incubator. Phagocytosis was monitored by phase contrast microscopy and expression of CaGto1 and CaGtt11 was analysed by fluorescence microscopy (Olimpus BZ51 microscope, with a U-MNUA3 filter).

Human neutrophils were isolated from peripheral blood of health human volunteers, using the S-Monovette 7.5 ml LH blood collection system (Sarstedt). Immediately after collection, Histopaque-1119 and 1077 reagents were used to enrich in cells of the granulocytic series following the manufacturer instructions (Sigma). Cells sedimenting on layer B were collected, checked at the microscope for red cell contamination, washed with isotonic phosphate buffered saline and then with RPMI 1640 medium, both of them preheated at 37ºC before use. In case of extensive contamination of the fraction with red blood cells, a short treatment (7 min at 37ºC) with RBCL solution (Red blood cell lysis: 0.83% NH_4Cl in 10 mM Hepes pH 7) preceded the two washes in order to provoke the lysis of the erythrocytes. Viability of the remaining cells (>99%) was checked by trypan blue dye exclusion. Then, 5x10^5 cells were spread in 35 mm plates and incubated for a period of 1 hour to allow recuperation and adhesion of the cells to the plates. Opsonized yeast cells (as for macrophage experiments, see above) in RPMI 1640 medium were added to the neutrophiles (ratio 1:20 to 1:40) and phagocytosis was monitored by phase contrast and fluorescence microscopy.

Results and discussion

GST genes in *C. albicans*

S. cerevisiae Gto1 protein sequence was used in a BLAST search for homologues in *C. albicans*. One ORF of this species (orf19.2613, in Candida Genome Database, CGD) codes for a translation product highly homologous to the three Gto proteins of *S. cerevisiae*. We therefore named orf19.2613 as *GTO1*, and the corresponding product as CaGto1. No other ORFs in the *C. albicans* genome code for proteins with significant homology to Gto1 proteins. However, many other fungal species have Gto relatives (Garcerá et al., 2006; Morel et al., 2009). A multiple alignment was done with the above four proteins plus the respective homologous products of *C. glabrata* gene CAGL0G02101 and *Schizosaccharomyces pombe* gene SPCC1281.07 (Fig. 1A). A large number of residues are conserved in the six proteins, including the CP pair essential for the activity of Omega-class GSTs (Whitbread et al., 2005). The CaGto1 sequence displays the closest homology with *S. cerevisiae* Gto2 and Gto3 and with the *C. glabrata* relative (Fig. 1B).

Similarly, a BLAST search with the *S. cerevisiae* Gtt1 protein sequence revealed four ORFs in the *C. albicans* genome whose products show some homology with the entire Gtt1 sequence. These ORFs are named orf19.6947, orf19.359, orf19.356 and 19.6998 in CGD, and have been respectively described as *GTT11*, *GTT12*, *GTT13* and *GTT14* in a recent study (Michán & Pueyo, 2009). We will employ the latter nomenclature in the present work. The four CaGtt proteins display higher homology among them than with *S. cerevisiae* Gtt1 and Gtt2, and CaGtt11/CaGtt12 and CaGtt13/CaGtt14 form two separate branches in a tree resulting from a multiple alignment (Supplementary Fig. S1). This therefore points to recent separate duplication events in the *C. albicans* genome involving *GTT11* and *GTT12* on one hand and *GTT13* and *GTT14* on the other. Interestingly, *GTT12, GTT13* and *GTT14* are in a region of chromosome 3 which seems to have been subjected to duplication followed by inversion (van het Hoog *et al*., 2007).

Enzyme activities of CaGto1 and CaGtt11

We determined the enzyme activities of CaGto1 and CaGtt11. These are the two *C. albicans* GSTs which have the more intense responses against a diversity of stresses (see below). Recombinant CaGto1 and CaGtt11 were purified by affinity chromatography from *E. coli* extracts for the *in vitro* determination of their activities.

CaGto1 had thiol oxidoreductase (with HED as substrate) and DHAR activities at levels slightly lower than the purified *S. cerevisiae* Gto2 protein (Fig. 2A), while no detectable activity was observed on CDNB (not shown). CaGtt11 displayed GSH-conjugating activity on CDNB and also had activity as GSH-dependent peroxidase, in both cases without significant differences with *S. cerevisiae* Gtt1 (Fig. 2B). Therefore, CaGto1 and CaGtt11 show similar activity patterns as the *S. cerevisiae* counterparts (Garcerá et al., 2006), confirming the existence of peroxidase activity in GSTs of the fungal GTT classes.

Response of *C. albicans* GST genes upon different stresses

GTO1 was expressed at very low (although detectable) levels in basal exponential growth conditions, as determined by Northern blot analysis. However, diverse environmental stresses caused significant upregulation of expression, which in most cases was transitory (Fig. 3). This was the case of oxidative stress by hydrogen peroxide and *tert*-butyl hydroperoxide, diamide [acting as an oxidant of sulfhydril groups (Kosower and Kosower, 1995)], CDNB [which as substrate of GSTs may deplete the GSH intracellular pool (Collinson & Grant, 2003)], or diethyl maleate, which diminishes the reduced vs. oxidized glutathione ratio. Osmotic stress by 0.6 M KCl or 1.2 M sorbitol also resulted in *GTO1* upregulation, as well as alkaline stress by KOH or calcium stress. In addition, iron deprivation or early stationary phase conditions also caused upregulation of expression, which was transitory in the latter case. On the contrary, cadmium caused transitory repression of *GTO1* expression (Fig. 3). Other conditions tested, such as heat shock from 25 to 39ºC in YPD rich medium, did not result in expression changes (data not shown). The pattern of *GTO1* expression in *C. albicans* upon treatment with different oxidants is most similar to *S. cerevisiae GTO2*, which displays the most intense response among the three *GTO* genes in this species (Barreto et al., 2006).

Expression of the four *C. albicans GTT* genes was also analyzed by Northern blot. Only *GTT11* showed detectable expression over background levels in exponential cells. This is in accordance with a recent study (Michán & Pueyo, 2009) that quantified mRNA molecules per cell in exponential cultures, in which it was demonstrated that *GTT11* mRNA levels were at least 100-fold higher than those of the other three *GTT* mRNAs. In addition, we could not detect upregulation of *GTT12, GTT13* or *GTT14* mRNAs upon oxidative, osmotic, alkaline or heat stresses (data not shown). In the case of *GTT11*, an intense transitory induction was observed upon the same stress conditions as for *GTO1* (Fig. 3). The exception was diethyl maleate, which induced *GTT11* expression only slightly over background levels. In contrast to *GTO1*,

expression of *GTT11* was upregulated by cadmium treatment (Fig. 3), similarly to *GTT1* and *GTT2* in *S. cerevisiae* (Barreto et al., 2006). In accordance with a previous study (Michán & Pueyo, 2009), *GTT11* became upregulated upon entry into stationary phase (Fig. 3). Induction of expression of *GTO1* and *GTT11* in early stationary phase cells may be related with the intracellular oxidant conditions generated in this population growth phase in yeast cells, which is reflected by the increase of carbonylated proteins (O'Brien et al., 2004; Reverter-Branchat et al., 2004).

The upregulation of both *GTO1* and *GTT11* expression upon oxidative and alkaline stresses is suggestive of some overlapping in the response to these stresses, as occurs in *S. cerevisiae* (Viladevall et al., 2004). In the budding yeast, a number of genes involved in reactive oxygen species detoxification and in redox regulation are upregulated by alkaline pH.

Role of Hog1 and Cap1 in the expression of *GTO1* and *GTT11*

In *C. albicans* cells growing in batch cultures, the oxidative stress response is specifically regulated by the Cap1 transcription factor (Zhang et al., 2000; Wang et al., 2006; Znaidi et al., 2009), which is homologue to the Yap1 factor playing a similar role in *S. cerevisiae*. In parallel, the MAP kinase Hog1 carries out a central role in the general stress response in *C. albicans* (Alonso-Monje at al., 2003; Smith et al., 2004; Enjalbert et al., 2006). Response to oxidants is part of the general stress response regulated by Hog1, although it does not seem to be a direct interplay between Cap1 and Hog1 (Enjalbert et al., 2006). Therefore, we analyzed the role of Cap1 and Hog1 in the upregulation of *GTO1* and *GTT11* upon the different stresses tested. Treatment with cadmium chloride, diethyl maleate or *t*-BOOH at the same concentrations employed in Fig. 3 did not result in significant changes of expression of *GTO1* or *GTT11* in the *C. albicans hog1* or *cap1* mutants compared to wild type cells (data not shown). However, other treatments resulted in significant differences of expression in the mutants. Thus, the response of *GTT11* to diamide stress was less intense in the *hog1* mutant (Fig. 4). Also, the response of *GTO1* to alkaline stress by KOH was moderately dependent on Cap1, a fact which may be surprising since it is commonly accepted that Cap1 is specific for the transcriptional response to oxidative stress. Analysis of the *GTO1* promoter (using the Regulatory Sequence Analysis Tools, http://rsat.ulb.as.be) revealed a YRE element (TTACTAA, recognition site by AP-1-like factors such as Yap1 or Cap1) at position -352. *GTT11* expression upon alkaline stress also depended on Hog1, but in this case the MAP kinase seems to carry out a repressor role, as gene expression was more intense in the mutant upon the stress (Fig. 4). The repressor function of Hog1 on gene expression in *C. albicans* had been

demonstrated before, both in basal growth conditions and upon oxidative stress (Enjalbert et al., 2006), but not upon alkaline stress. Interestingly, the response of *GTO1* to osmotic stress (0.6 M KCl) was more intense in the *cap1* and *hog1* mutants than in wild type cells (Fig. 4), indicating a repressor role for both Cap1 and Hog1 in such response. Such effect in the absence of Cap1 might be indirect in the case of *GTO1* during osmotic stress, since the Cap1 transcription factor does not seem to have a repressor function (Znaidi et al., 2009),.

In summary, *GTO1* and *GTT11* exhibit a complex dependence on both Cap1 and Hog1 which varies upon the stress applied, which is in accordance with the multiple pathways acting in the stress transcriptional response in *C. albicans* (Enjalbert et al., 2006).

Localization of CaGto1 and CaGtt11

A *C. albicans* strain was constructed which expressed a CaGto1 form tagged with GFP at the C-terminus, under the control of the own gene promoter. In exponential growth conditions, only a very tiny fluorescence signal was observed under the microscope. However, a diffuse clear signal was observed upon osmotic (KCl) and peroxide stresses, and in early stationary phase cultures and nutrient starvation conditions (Fig. 5A, and data not shown), paralleling the gene expression results. This observation points to a cytosolic location for CaGto1, as occurs with the Gto2 and Gto3 homologues of *S. cerevisiae* (Barreto et al., 2006).

A similar construction was made for CaGtt11, which was also expressed under its own promoter. Fluorescent signal was detected upon diamide and KCl stresses and in early stationary phase cells, but in this case a punctuated fluorescence pattern was detectable (Fig. 5B), which is characteristic of proteins associated to membrane vesicles. To confirm this membrane association of CaGtt11, we subfractionationated cell extracts from overnight cultures, followed by western blot analysis. Previously, we had observed by fluorescence microscopy that in these cells at the post-diauxic growth phase significant expression of CaGto1 and CaGtt11 already occurred (data not shown). The western blot analyses confirmed the association of a significant fraction of the CaGtt11 cellular pool to a particulate fraction (Fig. 5C). As a control, the CaGto1 protein remained at the supernatant. These observations are reminiscent of the described association of the CaGtt11 homologue in *S. cerevisiae*, Gtt1, to the endoplasmic reticulum (Choi et al. 1998).

Ex vivo expression of *C. albicans* GST proteins in phagocytic cells

The strains expressing the GFP-labelled derivatives of CaGto1 and CaGtt11 were employed for analyzing their expression inside phagocytic cells. Phagocytosis took place soon after (20-30 min) *Candida* cells have been added to RAW 264.7 murine macrophages. We observed upregulation of expression of both CaGto1 and CaGtt11 after internalization, and this was maintained during the first two hours following addition of the cells (Fig. 6A, and data not shown). After this time *Candida* cells began to form long mycelia and fluorescence disappeared.

In the case of freshly-prepared neutrophils, both CaGto1 and CaGtt11 became upregulated also during the first two hours inside the phagocytic cells (Fig. 6A). The fact that neutrophils carry out a more intense induction of an antioxidant response than the macrophages has been demonstrated at the transcriptional (Rubin-Bejerano et al., 2003; Fradin et al., 2005) and at the protein level (Enjalbert et al., 2007). This intense response is in accordance with the main role of neutrophils in the defence against *Candida* infections, considering the essential role of host-induced oxidative stress in the phagocytic attack against the fungal cell (Chauhan et al., 2006).

GTT14 is not upregulated in laboratory cultures in the different stress conditions tested (see above). However, its expression becomes increased in some conditions such as after long exposition to nutrient deprivation (Michán & Pueyo, 2009). In order to determine whether the CaGtt14 protein is expressed inside phagocytes, we intended to construct a version of the protein tagged at the C-terminus with the GFP sequence. However, this was not possible after repeated attempts, probably because incompatibility between the CaGtt14 and GFP sequences. As an alternative, we successfully constructed a strain (MML995) which expressed the GFP molecules under the control of the *CaGTT14* promoter (Table 1). In this way, we observed that this promoter was transitorily active in *Candida* cells inside macrophages and neutrophils, and that the kinetics of expression was similar to that of CaGto1 and CaGtt11 proteins (Fig. 6, and data not shown). This supports the possible importance of CaGtt14 as a defence mechanism during phagocytosis, in spite of the fact that the gene is not expressed at significant levels in normal laboratory conditions.

In conclusion, the present study points to CaGto1 and CaGtt11 (and probably also CaGtt11) as important components of the *C. albicans* program to counteract the oxidative burst occurring inside phagocytes, therefore contributing to virulence of the fungal cells. The fact that expression of CaGto1 and CaGtt11 is also responsive to other stresses which do not necessarily mimic the situation occurring inside the phagocytic cells may indicate that both GSTs may play additional defensive roles in other environments. CaGto1 and CaGtt11 display different enzyme activity patterns, in

the first case as redox regulators of protein thiol groups and in the second case as GSH-conjugating enzymes and peroxide detoxifiers. Therefore, it is predictable that their direct targets are different. Elucidating the respective targets may provide significant information on the virulence strategies used by *Candida* cells.

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Table 1. *C. albicans* strains employed in this study

Strain	Genotype	Source
CAI-4	*ura3*Δ::λ*imm*^{434}/*ura3*Δ::λ*imm*^{434}	Fonzi & Irwin (1993)
RM100	*ura3*Δ::λ*imm*^{434}/*ura3*Δ::λ*imm*^{434} *his1*Δ::*hisG/his1*Δ::*hisG-URA3-hisG*	Alonso-Monge et al. (2003)
RM1000	*ura3*Δ::λ*imm*^{434}/*ura3*Δ::λ*imm*^{434} *his1*Δ::*hisG/his1*Δ::*hisG*	Alonso-Monge et al. (2003)
CNC13	RM1000 *hog1*::*hisG-URA3-hisG/hog1*::*hisG*	San-José et al. (1996)
CCC1	RM1000 *cap1*Δ::*URA3/cap1*Δ::*HIS1*	San-José et al. (1996)
MML968	CAI-4 *RP10*::(pMM814) *URA3*	This work
MML970	CAI-4 *RP10*::(pMM833) *URA3*	This work
MML972	CAI-4 *RP10*::(pGFP) *URA3*	This work
MML995	CAI-4 *RP10*::(pMM843) *URA3*	This work

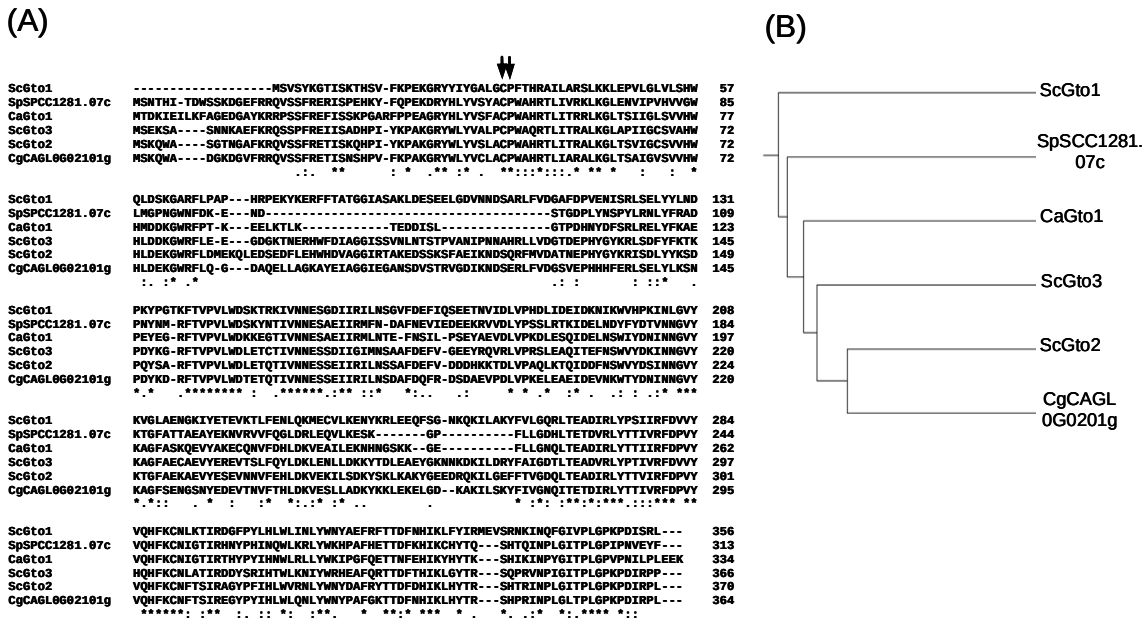

Fig. 1. Sequence comparison between CaGto1 and homologues from other fungal species. (A) Multiple alignment of the protein sequences using Muscle 3.7. (Edgar, 2004), from the European Bioinformatic Institute tools (http://www.ebi.ac.uk). The arrows indicate the two active site residues conserved in Omega-class GSTs. (B) Dendrograme tree from the previous alignment. Abbreviations: Ca, *Candida albicans*; Cg, *Candida glabrata*; Sc, *Saccharomyces cerevisiae*; Sp, *Schizosaccharomyces pombe*. UniProtKB database codes of the compared proteins: CaGto1, C4YL44; CgCAGL0G0201g, Q6FTJ1; ScGto1, P48239; ScGto2, P36156; ScGto3, Q04806; SpSCC1281.07c, O94524.

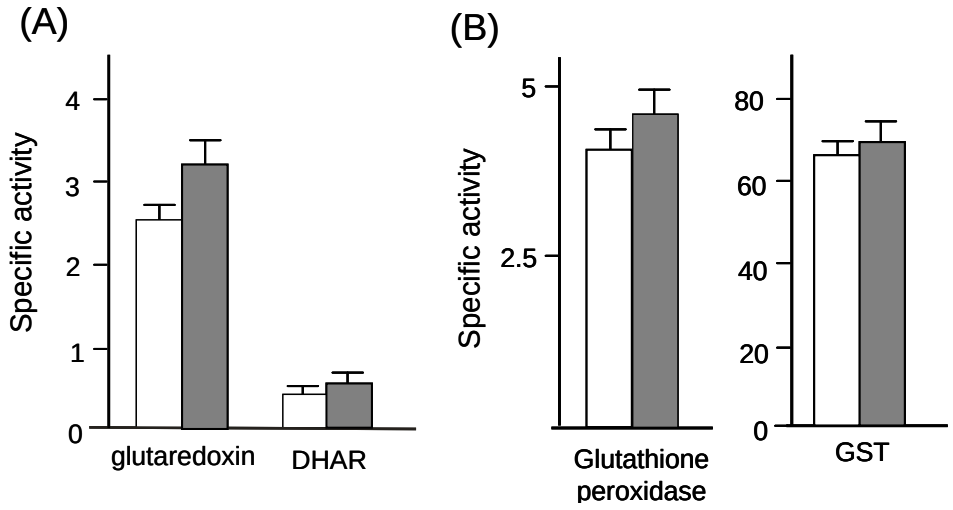

Fig. 2. Enzyme activities of purified CaGto1 (expressed from plasmid pMM803) compared to *S. cerevisiae* Gto2 (part A), and CaGtt11 (expressed from plasmid pMM801) compared to *S. cerevisiae* Gtt2 (part B). *S. cerevisiae* Gto2 and Gtt2 were purified as described in Garcerá et al. (2006). White boxes correspond to *C. albicans* proteins and grey boxes to *S. cerevisiae* proteins. Specific activities are expressed as μmols·min^{-1}·mg protein^{-1}, except for GST activity, which corresponds to nmols·min^{-1}·mg protein^{-1}. Bars indicate the mean (plus standard deviation) of three experiments.

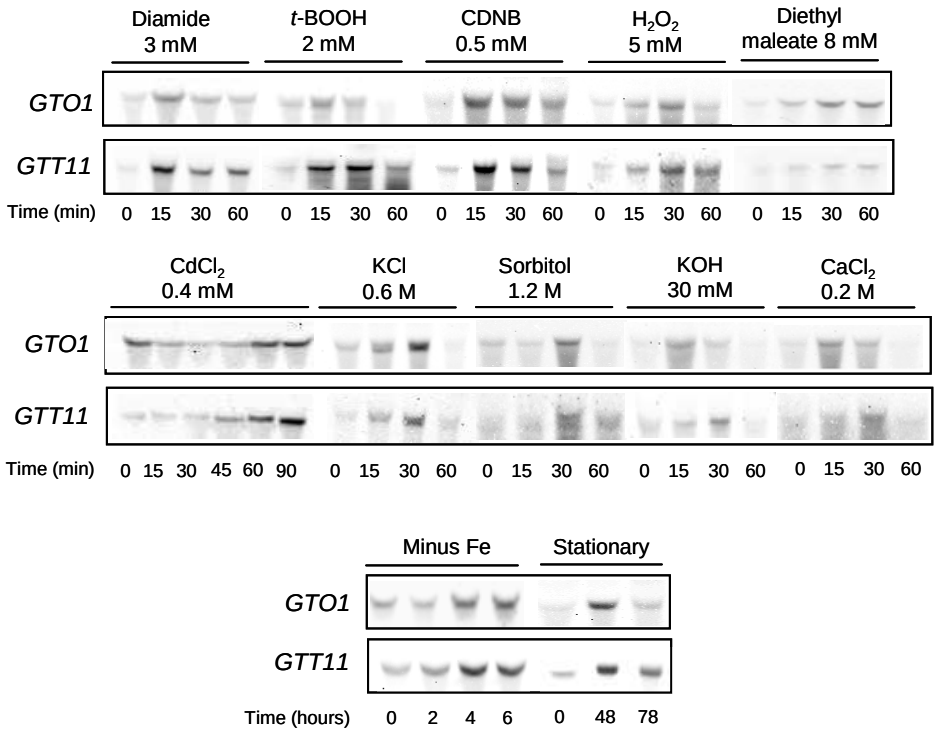

Fig. 3. Northern blot analysis of the expression of *GTO1* and *GTT11* under different stress conditions. *C. albicans* RM100 cells were grown exponentiallyat 30ºC in YPD medium to a concentration of about 1.5 x 10^7 cells/ml, except for cadmium treatment, in which case cells were grown in SC medium. Agents were added at time 0 at the indicated concentrations, and samples were taken at successive times for expression analysis. For iron depletion, ferrozine at 2mM concentration was added to the growing cells. Each lane contained 15 μg of total RNA. rRNA was visualized and employed as loading control in stained gels, without significant differences being observed.

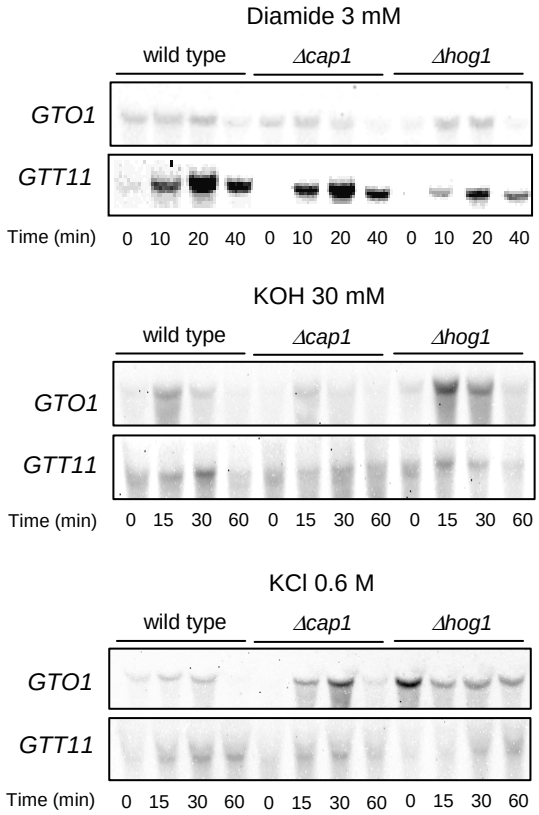

Fig. 4. Northern blot analysis of the expression of *GTO1* and *GTT11* under different stress conditions in wild type (RM1000), *Δcap1* (CCC1) and *Δhog1* (CNC13) cells. Exponentially growing cells at 30ºC in YPD medium (diamide and KCl treatments) or SC medium (cadmium treatment) were treated from time 0 in the indicated conditions, and samples were taken at successive times for expression analysis. Each lane contained 15 μg of total RNA. rRNA was visualized and employed as loading control in stained gels, without significant differences being observed.

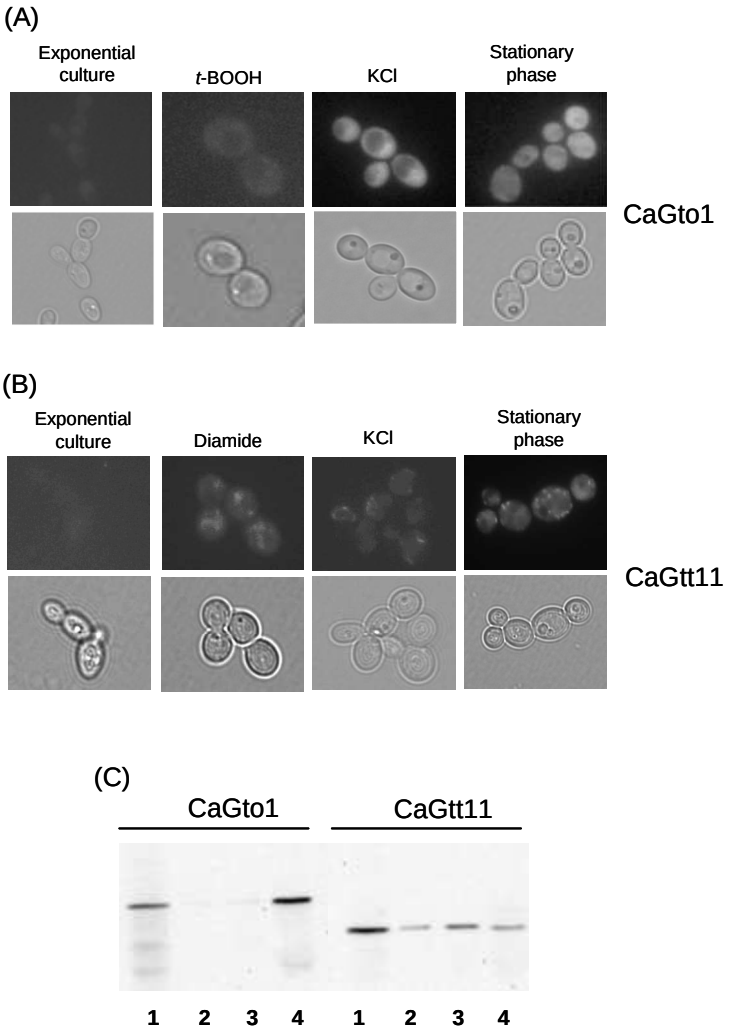

Fig. 5. Localization of CaGto1 and CaGtt11 in *C. albicans* cells. Untreated exponentially growing cells (YPD medium, 30ºC) of the MML968 strain expressing GFP-tagged CaGto1 under the own promoter (part A) and the MML970 strain expressing GFP-tagged CaGtt11 under the own promoter (part B), or the same strains subjected to different treatments, were observed by fluorescence microscopy (upper panels) and phase contrast microscopy (lower panels) in an Olimpus BZ51 microscope. The treatments employed were: *t*-BOOH: 2 mM, 30 min; KCl: 0.6 M, 30 min; diamide: 3 mM, 15 min: stationary phase: 48 h in YPD medium at 30ºC. Part C: Western blot analysis with anti-GFP antibodies after subcellular fractionation of cell extracts of the MML969 and MML969 strains grown overnight in YPD medium at 30ºC. The following fractions were analysed: total extract (lane 1), P13 (lane 2), P100 (lane 3) and S100 (lane 4). Lane 1 was loaded with 25 μg of protein, and the corresponding cell equivalents were loaded in the other lanes.

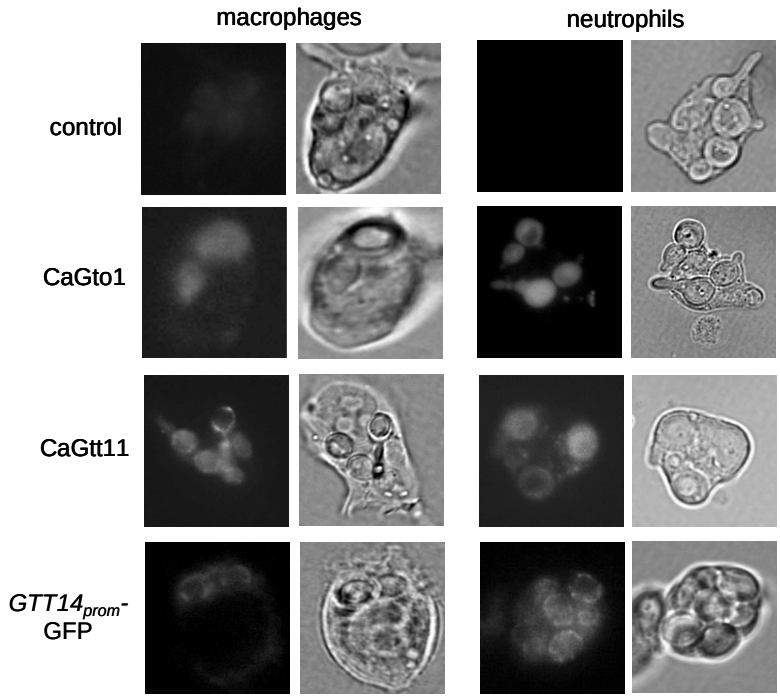

Fig. 6. *Ex vivo* expression of *GTO1, GTT11* and *GTT14* in phagocytic cells. Macrophages of the RAW264.7 line or neutrophiles prepared as described in Materials and Methods were exposed to opsonized *C. albicans* MML968 (expressing CaGto1-GFP from the own promoter in plasmid pMM814), MML970 (expressing CaGtt11-GFP from the own promoter in plasmid pMM833), MML995 (expressing GFP from the *GTT14* promoter in plasmid pMM843) or control MML972 cells (with the chromosomally-integrated pGFP vector). After 90 min incubation, images were taken by fluorescence microscopy (left) and phase contrast microscopy (right).

(A)

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ScGtt2   --------MNGRGFLI-YNGGEKMKQKMIIYDTPAGPYPARVRIALAEKNMLSSVQFVRINL-WKGEHKKPEFLAKNY    68
ScGtt1   --------MSLPIIKVHWLDHSRAFRLLWLLDHLNLEYEIVP--------------YKRDAN-FRA---PPELKKIHP    52
CaGtt11  --------MSDTKIIVHWLNYSRSQRVIWLLEELNIPFELKV--------------YLRNKQ-FRA---PKELENVHP    52
CaGtt12  --------MSDSKIILHWLNYSRSQRVLWLLGELNIPFELKV--------------YLRNKE-FRA---PKELENVHP    52
CaGtt13  ----MEGKGCDDRFILHWLDDSRTHRILWLLEILQLDYEVKI--------------YLRHPETWRG---PLQLFDVHQ    61
CaGtt14  MTMDTPNHQLEDRFILHWLDDSRSHRILWILDLLNLDYEVKI--------------YLRHPETWRG---PLQLFDAHQ    57
                    : : : . ..  . : :      :                 : *    :..    ::   :

ScGtt2   SGTVPVLELDDGT-----LIAECTAITEYI-DALDGTPTLTGKTPLEKGVIHMMNKRAELELLDPVSVYF-------H   133
ScGtt1   LGRSPLLEVQDRETGKKKILAESGFIFQYVLQHFDHSHVLMSEDADIADQINYYLFYVEGSLQPPLMIEFILSKVKDS   130
CaGtt11  LGKSPVIEVIDTKTGESEVIAETGHIFNYILSNYDTTNILTPANRKLQNQVDYFLHYAEGTLQ-PNLVALLVHGFAKQ   129
CaGtt12  LGKSPVIEVIDSKTGKSEIIAETGHIFNYILSNYDTTNILIPFNRDLQSQVDYFLHYTEGTLQ-PKLVALMVHGVAKK   129
CaGtt13  LGKAPVLEIIFGDGRPPIKITESGFIIQYLLRVYDKENILNPISQEQQLEVDYYLHYAEGSLQ-HIQMALLINSVAKH   134
CaGtt14  LGKAPVLEVIFGDGRPPIKISESGFIIQYLLRYYDCQNILYPANLDQQLEVDYYLHYSEGSLQ-HIQMALLINSSAKH   138
          *  *::*:          ::*   * :*:    *    *          :       *  *     : :

ScGtt2   HATPGLGPEVELYQNKEWGLRQRDKALHGMHYFDT-----VLRERPYVAGDSFSMADITVIAGLIFAAIVKL------   200
ScGtt1   GMPFPISYLARKVADKISQAYSSGEVKNQFDFVEG----EISKNNGYLVDGKLSGADILMSFPLQMAFERKF------   198
CaGtt11  QAPFGTKFLMGLLVNGIDSMFYIPELKKNLNYLEDIMRKQHENGSNYFVGDKLSGADIILEFPVITNIFQNKRGAEQL   207
CaGtt12  KAPFGARFLMGLLMGGIDDAFYIPDLKKNLKYLENIIHKQHEKGSKYFVGDKLSGADIILEFPVITNIFQNKRGAEQL   207
CaGtt13  VAPFATKAVVKIITKAINNGYYKHEWYLNFQYLED---RLAQNGTGFFVGNKLTGADVILSFPVYENVFDNPGGVREI   209
CaGtt14  IAPFATKSVVKLVTKAINNGYYKHEWFLNMKYLED---RLEQNGTGFFVGDKLSGADVILSFPIYENVFDNLEGTKEI   213
           .                    .    : :.:       .   :.....:: **: :   :     :

ScGtt2   -----QVPEECEALRAWYKRMQQRPSVKKLLEIRSKSS----------------   233
ScGtt1   -----AAPEDYPAISKWLKTITSEESYAASKEKAR-------ALGSNF------   234
CaGtt11  GAG--DVEKEYPHLNQWAEDIKKEPKYIKAQELVAKHE----TVKPNI------   249
CaGtt12  GAG--DVEKEYPHLNQWAEDIKKEPKYIKAQELVAKHE----TVKPNI------   249
CaGtt13  CGEKRDLRKVYPHLAKWSRMIKNNPTYKRVTEMMNEEVEDLIAMNPRFDYGKEK   263
CaGtt14  LGDKVNIKKMFPNLFNWSRMIKNNLSYKKITELMNEEVEDLIALNPRFDYGREK   267
                :   :  *   : .  .     *
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(B)

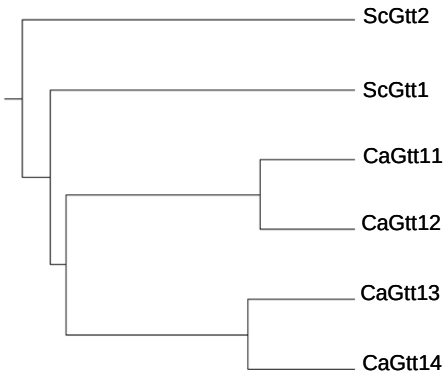

Fig. S1. Sequence comparison between CaGtt proteins and the *S. cerevisiae* homologues ScGtt1 and ScGtt2. (A) Multiple alignment of the protein sequences using Muscle 3.7 (Edgar, 2004), from the European Bioinformatic Institute tools (http://www.ebi.ac.uk). (B) Dendrograme tree from the previous alignment. Abbreviations: Ca, *Candida albicans*; Sc, *Saccharomyces cerevisiae*. UniProtKB codes of the compared proteins: CaGtt11, Q59SS2; CaGtt12, Q5AEA9; CaGtt13, Q5AEB2; CaGtt14, Q59YS5; ScGtt1, P40582; ScGtt2, Q12390.