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*Candida albicans*의 분화에서 glutathione reductase의 역할

Role of glutathione reductase in the differentiation of *Candida albicans*

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Role of glutathione reductase in the differentiation of *Candida albicans*

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ABSTRACT

Glutathione reductase adequately maintains the glutathione level in a reduced state to regulate redox state. It was evidently confirmed that glutathione is required for cell growth and division, and its biosynthesizing-enzyme deficiency causes methylglyoxal accumulation. However, experimental evidence for reciprocal relationships between Cph1-/Efg1-mediated signaling pathway regulation and methylglyoxal production exerted from glycolysis pathway by glutathione reductase on yeast morphology, and the origin of methylglyoxal production remain unclear.

In this study, glutathione reductase (*GLR1*) mutants were used to examine aspects of pathological and morphological alterations in *Candida albicans*. These were proved by observations of cellular susceptibility to oxidants and thiols, and measurements of methylglyoxal, reactive oxygen species and glutathione content in hyphal-inducing conditions mainly through the activity of *GLR1*-overexpressing cells. Additionally, the transcriptional and translational levels of bioenergetic enzymes and dimorphism-regulating protein kinases were examined in the strain.

As expected, the *GLR1*-deficient strain was non-viable when *GLR1* expression under the control of a *CaMAL2* promoter was conditionally repressed even with various exogenous thiols. During filamentation, *GLR1*-overexpressing cells displayed non-hyphal growth and exhibited resistance against oxidants and the cellular methylglyoxal decrease significantly, which concomitantly increased expressions of genes encoding bioenergetic enzymes, including fructose-1,6-bisphosphate aldolase (FBA1), glyceraldehyde-3-

phosphate dehydrogenase (TDH3), and alcohol dehydrogenase (ADH1), with remarkable repression of Efg1-signaling cascades.

Herein, *GLR1* repressed Efg1-mediated signal transduction strictly to reduce morphological switching and virulence by maintaining the basal level of methylglyoxal following the high gene expressions of glycolytic enzymes and *ADH1*. The Efg1-mediated down-regulatory mechanism by *GLR1* expression has possibilities to involve in other complex networks of signal pathways, especially glycolysis.

Keywords: Glutathione reductase; glutathione; methylglyoxal; fructose-1,6-bisphosphate aldolase; Efg1-mediated cyclic AMP/protein kinase A pathways; *Candida albicans*

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LIST OF ABBREVIATIONS

MG	methylglyoxal
GCS	γ-glutamylcysteine synthetase
GSH	reduced glutathione
GSSG	oxidized glutathione
ORF	open reading frame
GLR1	*C. albicans* glutathione reductase 1
FBA1	*C. albicans* fructose-1,6-bisphosphate aldolase 1
ADH1	*C. albicans* alcohol dehydrogenase 1
TDH3	glyceraldehyde-3-phosphate dehydrogenase
ALO1	D-arabinono-1,4-lactone oxidase
EAPX1	erythroascorbate peroxidase
EASC	D-erythroascorbic acid
ROS	reactive oxygen species
SOD	superoxide dismutase
HPLC	high-performance liquid chromatography

I. INTRODUCTION

1. *Candida albicans*

Candida albicans is found in the normal gastrointestinal flora and the oral mucosa of most healthy humans (Soll, 2002). It is also known as the major fungal pathogen of humans, causing serious systemic infections in immunocompromized patients including HIV and cancer patients. *C. albicans* has diverse cell morphogenesis from blastospore form to filamentous form including a thread-like true hypha, germ tubes, and pseudohyphae (Mitchell, 1998). The major morphological difference between true hyphae and pseudohyphae is that true hyphae have a persistent width and narrow filaments with no constriction at the septa whereas pseudohyphae have the variable width and relatively thick filaments with constriction at the septa (Sudbery *et al.*, 2004). *C. albicans* has no known sexual cycle and is at least diploid (Scherer and Magee, 1990).

1.1. Environmental cues affect *C. albicans*

Morphology of *C. albicans* can be reversible depends on environmental conditions including pH, temperature and media: a temperature above 35℃ (Saraswat *et al.*, 2016), neutral pH, poor source of carbon and nitrogen, some amino acid mixture (Lee's medium), and nutrient broth (spider medium) also induce a hyphal development as Scheme 1. This feature is necessary for *C. albicans* to survive within a host. In general, yeast form predominates during mucosal colonization in a normal host but when a host's immune system defects, hyphae are induced from yeast cells (Berman and Sudbery, 2002; Brown Alistair J.P and Gow Neil A.R 1999).

1.2. Various virulence factors of *C. albicans*

Potential virulence factors for *C. albicans* are as follow: yeast-hyphal morphogenesis (Bertram *et al.*, 1996; Brown Alistair and Gow Neil, 1999), adhesion to host cells or ligands of host cells include agglutinin-like proteins, transglutaminase and integrin-like proteins (Calderone and Fonzi, 2001; Sundstrom *et al.*, 2002) and production of extracellular hydrolytic enzymes (Hube and Naglik, 2001). Also, Hwp1 (Hyphal wall protein 1), hyphae and germ tube specific gene, was isolated by the differential screen (Staab *et al.*, 1996). Hwp1 is a well-known outer surface mannoprotein, which is found on surfaces of germ tube, but not yeast in *C. albicans*. Hwp1 is known as a substrate for the mammalian transglutaminase. The *HWP1* mutant strains were avirulent, but a disruptant in single allele or wild-type strains have remained lethal in a hematogenous disseminated murine model (Sundstrom *et al.*, 2002). The antioxidant defense systems are also thought to be pivotal for this organism both to resist the host immune response and to exert its virulence. These assertions are supported by previous reports that D-erythroascorbic acid (EASC)- (Huh *et al.*, 2001; Kwak *et al.*, 2015), or copper-/zinc-containing superoxide dismutase (*SOD1*)-deficient *Candida* cells (Hwang *et al.*, 2002) showed defective *in vivo* hyphal growth and reduced virulence. Moreover, the mitochondrial manganese-containing SOD2 has been demonstrated to be required for *C. albicans* to endure various external stresses, such as oxidants, high salts concentration, ethanol, or hyperoxic conditions (Hwang *et al.*, 2003).

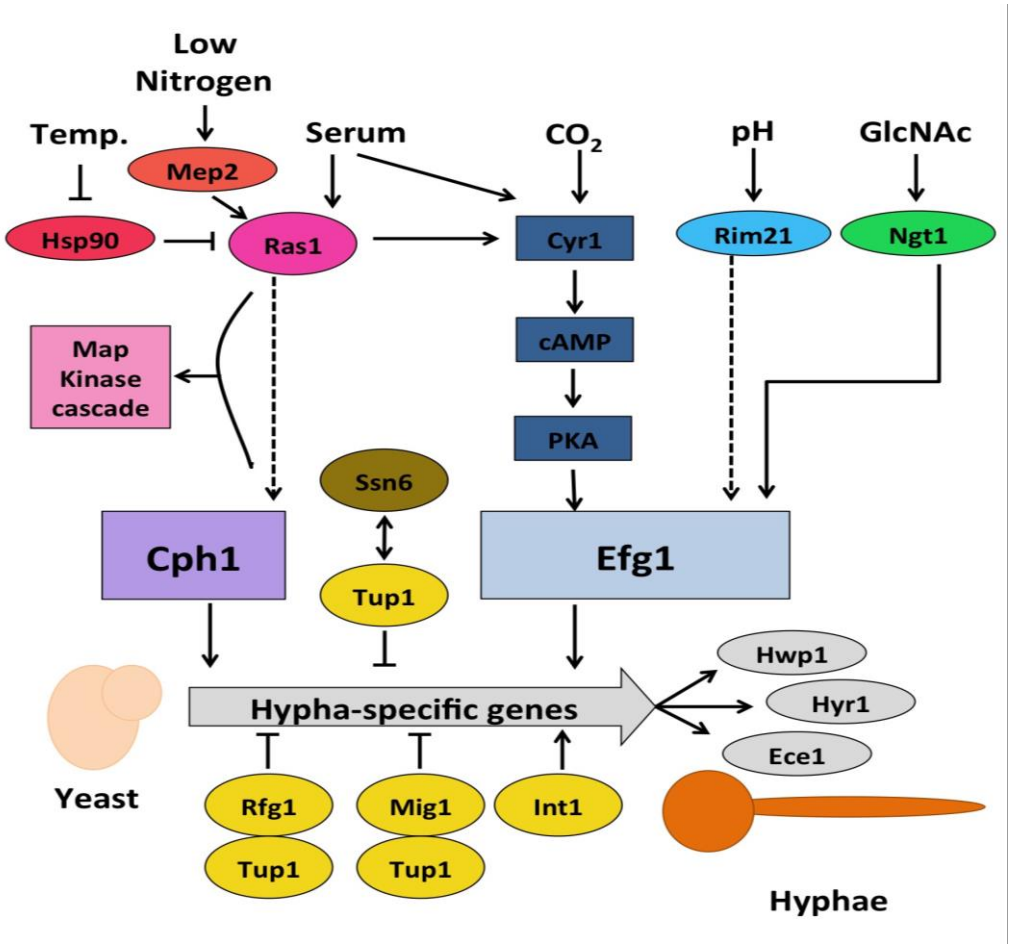
1.3. Signal transduction pathways

Considering the significance of causative mucosal and cutaneous infections in immunocompromized individuals derived from *Candida* pathogenicity (Gow

et al., 2002), the morphological conversion from yeasts to hyphae, which is well conserved of various types of indicators, including a Cph1-mediated mitogen-activated protein kinase (Liu *et al.*, 1994) and an Efg1-mediated cyclic AMP/protein kinase A pathways (Ernst J. F., 2000), is closely associated with the virulence (Brown A.J.P. and Gow N.A., 1999). Furthermore, through Mitogen-Activated Protein Kinase (MAPK) pathways, the RAS pathways, the calcium-calcineurin pathway and amino acid sensing and signaling pathways (Biswas *et al.*, 2007; Rispail *et al.*, 2009; Zhao *et al.*, 2007), dimorphism of *C. albicans* is regulated (Szent-Györgyi *et al.*, 1967). Moreover, the exogenous MG inhibits human leukemia 60 cells, leading to apoptosis and G1 arrest (Kang *et al.*, 1996). MG also triggers the activation of the stress-activated protein kinase cascade by acting as a signal molecule via a response regulator in the fission yeast *Schizosaccharomyces pombe* (Takatsume *et al.*, 2006). Considering cellular energy metabolism and its regulation, MG inactivates glyceraldehyde-3-phosphate dehydrogenase, a key glycolytic enzyme, causing inhibition of glycolysis in Ehrlich ascites carcinoma cells (Biswas *et al.*, 1997).

1.4. Biosynthesis and degradation of methylglyoxal

MG can be found from various organisms as a side-product of different metabolic pathways including glycolysis, photosynthesis, lipid peroxidation and oxidative degradation of glucose and glycated proteins (Allaman *et al.*, 2015; Kalapos, 1999; Thornalley *et al.*, 1999). MG is produced by certain bacteria as a byproduct of glycolysis through methylglyoxal synthase including Group A *Streptococcus* and *Escherichia coli* (Ferguson *et al.*, 1998; Thornalley *et al.*, 1999; Zhang *et al.*, 2016). As Scheme 2, MG can be synthesized by MG synthase. It was first reported from *E. coli.* (EC.4.2.99.11) (Hopper and Cooper



Scheme 1. Regulation of *C. albicans* morphogenesis through various signaling pathways. Various environmental cues trigger filamentous signaling pathways in *C. albicans*. Two major signaling pathways, Cph1-mediated MAPK pathway, and Efg1-mediated cAMP pathway activate filamentous growth (adapted from (Gow et al., 2012; Hwang et al., 2003; Sudbery et al., 2004)).

1971). The activity of MG synthase is controlled by inorganic phosphate (P_i), and MG synthase affects cellular methylglyoxal concomitant with glucose metabolism (Hopper and Cooper, 1972). Cytochrome P450s (EC.1.14.14.1) in acetone metabolism is another enzyme, which converts acetone into MG using NADPH and H^+(Casazza *et al.*, 1984; Koop and Casazza, 1985). The last enzyme that can produce MG is amine oxidases. Amine oxidases control glycolytic bypass, acetone metabolism, and amino acid breakdown (Kalapos, 1999). Simply, amine oxidase converts aminoacetone into methylglyoxal from threonine and glycine bio-degradative mechanism (Lyles, 1996). On the other hand, Thornalley (2003) strongly argues that MG can be produced non-enzymatically from glyceraldehyde-3-phosphate (G-3-P) and dihydroxyacetone phosphate (DHAP) by eliminating phosphate. However, there is no elusive piece of evidence to support his theory.

It is not exclusively studied why MG is produced, regulated and maintained. However, maintaining of MG homeostasis is necessary. So far, MG can be degraded by the glyoxalase system and non-glyoxalase system. The glyoxalase system is the major pathway of degradation of MG present in the cytosol of cells and cellular organelles, particularly mitochondria, in living systems (Kaur C. *et al.*, 2014; Li, 2016; Thornalley, 2003).

The glyoxalase system consists mainly of two enzymes, Gly I and Gly II. Spontaneously, hemithioacetal is formed between glutathione and MG and then converts to S-D-lactoylglutathione by Gly I. Furthermore, Gly II catalyzes the hydrolysis of S-D-lactoylglutathione to form D-lactate and glutathione (Hasim *et al.*, 2014; Thornalley, 2003). Hence, cellular glutathione is important to detoxify MG. Deficiency of glutathione leads to accumulation of MG, cell damage and growth defects (Choi *et al.*, 2008; Kwak *et al.*, 2014).

A non-glyoxalase system the can degrade MG. Because MG contains two functional groups (aldo- and keto-), it can either oxidized or reduced by aldose/aldehyde reductase or aldo-keto reductase using NADH or NADPH to form acetol, lactaldehyde, and pyruvate (Hossain *et al.*, 2012; Kaur *et al.*, 2014). Also, MG dehydrogenase, MG reductase, and aldehyde dehydrogenase catalyze the conversion of MG into pyruvate as the final product. Pyruvate enters into the tricarboxylic acid cycle. Recently, NAD^+-linked alcohol dehydrogenase (ADH1) is reported to catalyze MG oxidation and reduction in *C. albicans* by alternating NAD^+/NADH ratio (Kwak *et al.*,2014).

1.5. Free radical generation by methylglyoxal

MG production strongly correlates with the generation of superoxide radicals and reactive oxygen species (ROS) (Du *et al.*, 2001). Moreover, MG-amine, MG-amino acid, and MG-protein adduct result in producing biologically active free radicals (Lee *et al.*, 1998; Szent-Györgyi and McLaughlin, 1975; Yim *et al.*, 1995). MG-derived advanced glycation end-products (Ramasamy *et al.*, 2012) act as a causative factor of hyperglycemic damage to cells (Song and Schmidt, 2012). Additionally, the MG-induced embryonic malformations along with the strongly reduced viability might also be ascribed to energy depletion associated with mitochondrial dysfunction (Amicarelli *et al.*, 2001). Previously it was noted that an increase in superoxide anions was observed during the glycation reaction of amino acids by MG followed by the generation of two other types of free radicals, including the cross-linked radical cation and MG radical anion (Yim *et al.*, 1995). The MG autoxidation and its ability to generate mutagenic H_2O_2 have been proven by the oxygen consumption or the ferricytochrome c reduction measurement (Thornalley *et al.*, 1984).

2. Glutathione

2.1. Properties of glutathione

Glutathione ($_{L}$-γ-glutamyl-$_{L}$-cysteinylglycine, GSH) plays a pivotal role in various biological processes, including electrophile detoxification, steady-state thiol content maintenance, enzyme activity modulation and antioxidative defenses (DeLeve and Kaplowitz, 1991; Hutter *et al.*, 1997; Meister, 1988; Meister A. and Anderson M. E., 1983) and is known as major intracellular thiol-disulfide redox buffers that are serves as a cofactor for many antioxidant enzymes. GSH is synthesized in two steps by γ-glutamylcysteine synthetase (GCS) and glutathione synthetase (Scheme 3). The first step catalyzed by GCS is condensation of glutamic acid to cysteine and the rate-limiting step of GSH synthesis. Then, glutathione synthetase catalyzes the formation of GSH from glutamyl-cysteine and glycine. Also, GCS is subject to feedback inhibition by GSH (Meister A. and Anderson M. E., 1983). Maintaining the high intracellular ratio of GSH to GSSG, oxidized glutathione (GSSG) is efficiently reduced by GSH reductase.

2.2. Functions of glutathione

Since GSH is a thiol source of reducing equivalent, it has been demonstrated that GCS-null mutants of *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe,* and *C. albicans* display GSH auxotrophy (Baek *et al.*, 2004; Chaudhuri *et al.*, 1997; Grant *et al.*, 1996) and GSH deficiency leads to apoptosis (Baek *et al.*, 2004). In addition to that, *Dictyostelium discoideum* GCS-null mutant exhibits the G1 cell cycle arrest and developmental defect (Kim *et al.*, 2005).

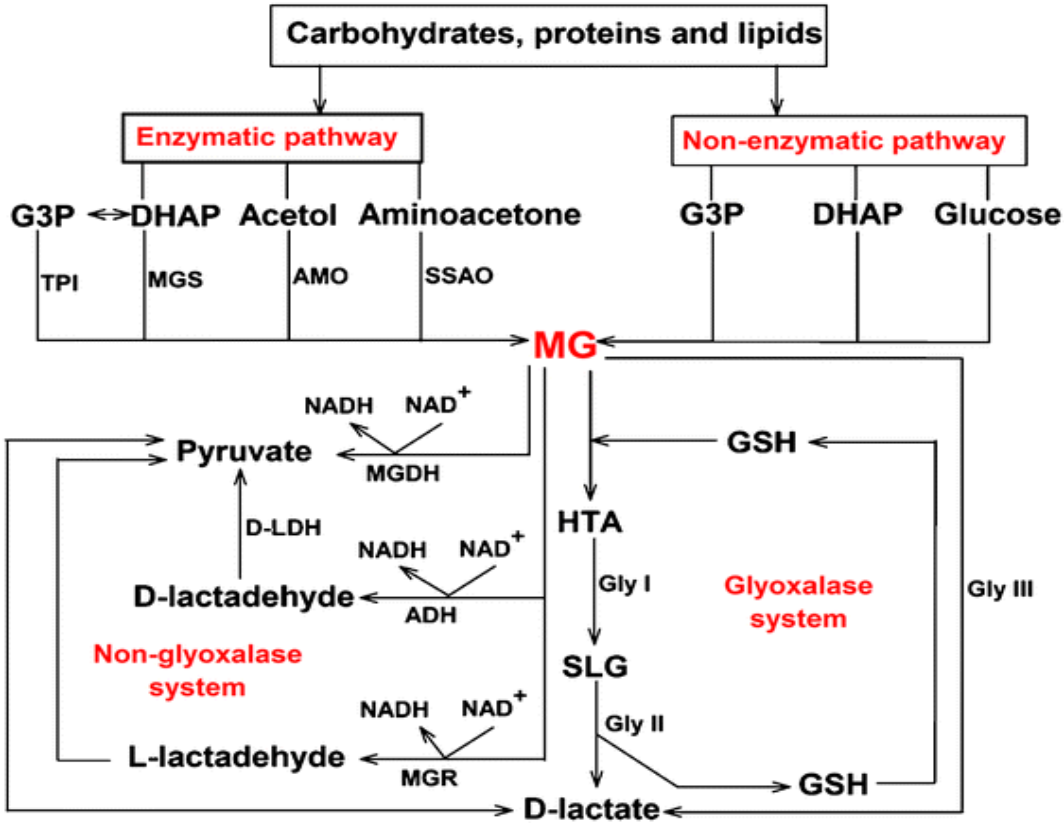
The unique role of GSH is an efficient radical scavenger. Numerous

reported studies are showing that radical scavenging by GSH produces superoxide and via dismutation to hydrogen peroxide (Forman, 2016; Winterbourn, 2016). Moreover, in *C. albicans* and *D. discoideum*, the experimental pieces of evidence prove that exogenous GSH reduces cellular MG contents and cellular GSH and MG are reciprocally related (Kwak *et al.*, 2014; Kwak *et al.*, 2016).

2.3. Glutathione reductase

As mentioned, GSH reductase (GR) reduces GSSG to GSH. This process serves to maintain a high intracellular ratio of GSH to glutathione disulfide, GSSG (Gill *et al.*, 2013). While separated from the γ-glutamyl cycle, patients with GR deficiency have been reported to exhibit early stage of cataract formation and favism due to an almost complete dimerization domain defect resulting unstable and inactive GR (Kamerbeek *et al.*, 2007; Meister, 1988; Meister A. and Anderson M.E., 1983).

In *E. coli*, the *GR*-deficient mutants have growth rates similar to the wild-type strains, and the ratio of GSH/GSSG was not significantly altered, suggesting that GSSG can be reduced independently of GR (Tuggle and Fuchs, 1985). This reduction can be accomplished by either the glutaredoxin (Grx) or thioredoxin (Trx) system (Russel and Holmgren, 1988). Despite being dispensable for cell growth, the activity of GR was crucially engaged in defenses against oxidative stresses in *S. cerevisiae* (Grant *et al.*, 1996). The *glr1* mutants that lack the genes for both *Trx1* and *Trx2* were nonviable (Muller, 1996). Thus, yeast cells seem to require the presence of either the GSH- or the Trx-dependent reducing systems for cell viability (Carmel-Harel and Storz, 2000; Grant, 2001).



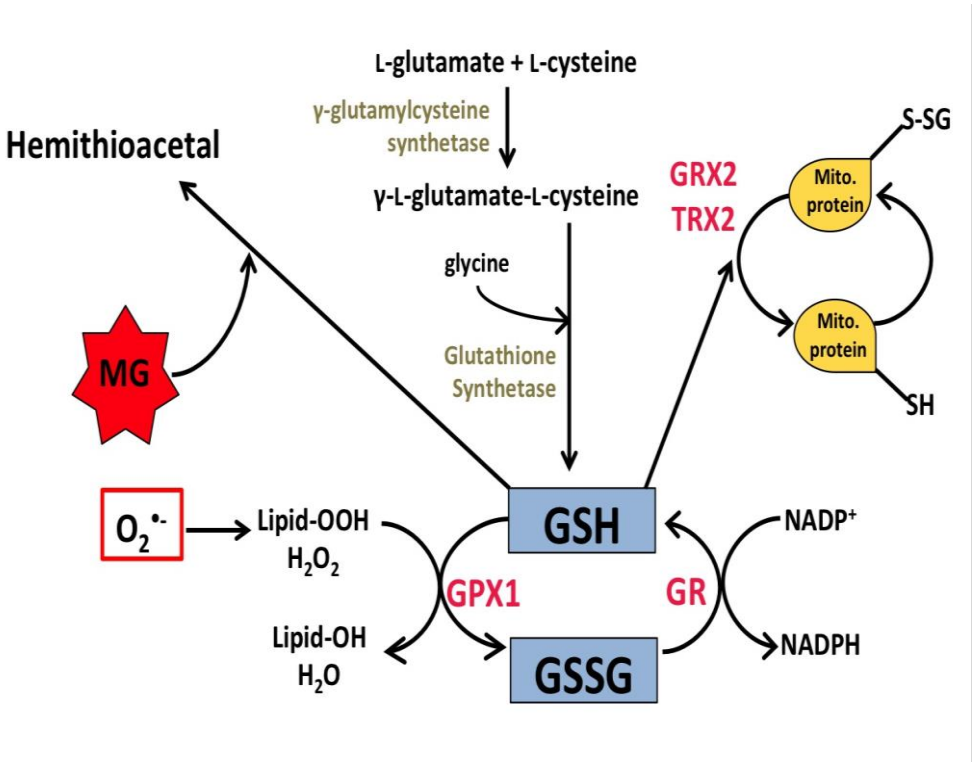
Scheme 2. Scheme of how methylglyoxal is synthesized and degraded from Li (2016).

MG: methylglyoxal; G3P: glyceraldehyde-3-phosphate; DHAP: dihydroxyacetone phosphate; TPI: triose phosphate isomerase; MGS: methylglyoxal synthase; AMO: acetol/acetone monooxygenase; SSAO: semicarbazide-sensitive amine oxidase; MGDH: methylglyoxal dehydrogenase; D-LDH: lactadehyde dehydrogenase; ADH: alcohol dehydrogenase; MGR: methylglyoxal reductase; GSH: glutathione; HTA: hemithioacetal; SLG: S,D-actoyl glutathione Gly: glyoxalase.

Unlike *E. coli* and *S. cerevisiae*, the *GR*-deficient *S. pombe Δpgr1* were not viable, inferring that GR activity is an essential factor for cell growth (Lee *et al.*, 1997), because the strain failed to grow even in the presence of Trx (Cho *et al.*, 2001) and Grx (Kim *et al.*, 1999). These evidences imply that GR may play different roles in different organisms. In the case of the *GLR1* (orf19.4618) encoding GR of *C. albicans*, the Glr1 seemed to be a member of the pyridine nucleotide-disulfide oxidoreductase family of flavoenzymes, which include dihydrolipoamide dehydrogenase, thioredoxin reductase, trypanothione reductase, and mercuric reductase (Williams, 1995).

Recently, the group of Alistair Brown (Tillmann *et al.*, 2015) constructed *Glr1* disruptant fundamentally based on amino acid auxotroph strain, BWP17 (*ura3Δ::imm434/Δura3Δ::imm434,his1Δ::hisG/his1Δ::hisG,arg4Δ::hisG/arg4 Δ::hisG*). Also, they mainly addressed the robust anti-stress defense system induced by nitrosative, oxidative, and formaldehyde stress. The NADPH-dependent Glr1 and S-nitrosoglutathione reductase, Fdh3, which detoxifies S-nitrosoglutathione to GSSG and NH_3, were contributed to different roles in GSH-dependent recycling systems that can manage in redox homeostasis, stress adaptation, and virulence in *C. albicans* (Tillmann *et al.*, 2015).

Previously, Baek (2003) described the role of *C. albicans GLR1* (Scheme 4). Baek (2003) generated *GLR1*-null mutants by placing the remaining *GLR1* allele under the control of *CaMAL2* promoter as suggested by Backen (Backen *et al.*, 2000) to avoid the effect of arginine on an Efg1p-dependent yeast-to-hypha switch (Ghosh *et al.*, 2009). The *GLR1*-overexpressing mutant was tested against various oxidative stress including diamide, *t*-butyl hydroperoxide, hydrogen peroxide and menadione showed strong resistance to it as seen in Scheme 4 (Baek, 2003). In addition to that,



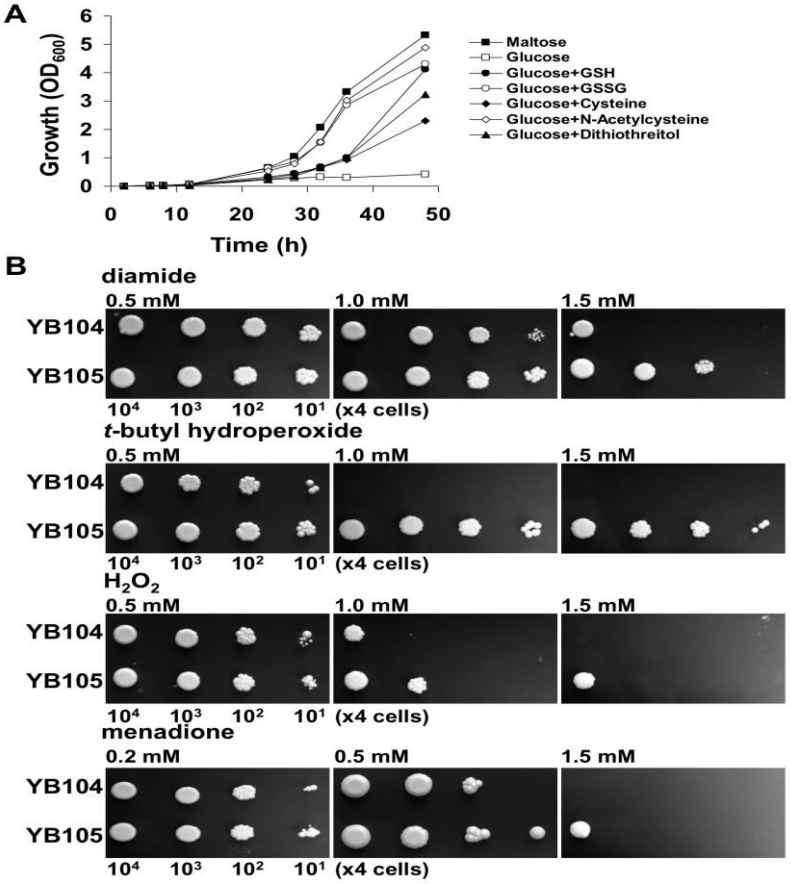
Scheme 3. Synthesis of glutathione (GSH) and its redox cycle.

GSH: reduced glutathione; GSSG: oxidized glutathione; GPX1: glutathione peroxidase; GR: glutathione reductase; GRX2: glutaredoxin; TRX2: thioredoxin; MG: methylglyoxal Mito. Protein: mitochondrial protein glutathionylation (adapted from Ribas *et al.*, 2014).

GLR1-overexpressing mutant displays attenuated virulence in a mouse model compared to reference strains.

3. Aims of Study

Herein, the minimal production of MG and reactive oxygen species (ROS) dedicated due to highly-steady state GR activity of non-filamentous, budding yeast-like *GLR1*-overespressing *Candida* cells. The results were in sharp contrast to MG- and ROS-accumulating GSH-depleted cells, including *Candida GCS1*-, *ADH1*-, or *EAPX1*- and *Dictyostelium* ornithine decarboxylase (*odc*)- or *gcsA*-deficient strains for characterization of MG scavengers (Choi *et al.*, 2008; Kim *et al.*, 2005; Kwak *et al.*, 2014; Kwak *et al.*, 2016; Kwak *et al.*, 2015). Thus, this current study verified functional roles of the *GLR1* overexpression both in the redox regulation modulated by GSH biosynthesis enhancement and incidental MG detoxification and especially in other regulatory gene expressions that can be affected by GR activities, noticeably during filamentation and bioenergetic processes conveyed by their phenotypic and pathogenic alterations. I thereby focused on the impact of *GLR1* overexpression mainly on morphology and virulence in *C. albicans*. However, GSH itself was not found to affect morphogenesis and virulence directly neither exogenous nor endogenous GSH. Most importantly, the *GLR1* expression down-regulated virulence- and hypha-specific genes through an Efg1-dependent pathway. The results were possibly linked with the gene expressions of glycolytic enzymes, including fructose 1,6-bisphosphate aldolase (*FBA1*, orf19.4618), glyceraldehyde-3-phosphate dehydrogenase (*TDH3*, orf19.6814), and *ADH1* (orf19.3997) (Kwak *et al.*, 2014) concomitantly accompanying the significant decrease in cellular MG, prominently during *C. albicans* dimorphic switching.



Scheme 4. Effect of thiols on the growth of *GLR1*-deficient mutants and induction of GLR1 expression by various stresses (A) The YB103 cells were inoculated from a saturated grown seed culture to an OD_{600} of near 0.01 in minimal medium containing maltose, glucose or glucose supplemented with various thiols (each at 1 mM). The growth rate was monitored by measuring the optical density at 600 nm. (B) Exponentially growing cells of YB104 and YB105 (were spotted as serial 1/10 dilution on solid minimal medium containing each chemical at the indicated concentration. Plates were incubated at 28°C for 3 days**.** (adapted from Baek, 2003).

II. Materials and Methods

1. Materials

1.1. Yeast strains and growth conditions

The *C. albicans* strains used in this study are listed in Table 1. For the routine growth of *Candida* cells containing disrupted genes, cells were cultured in YPD (1% yeast extract, 2% peptone, 2% glucose) , minimally defined SD (2% glucose, 0.5% ammonium sulfate, 0.17% yeast nitrogen base without amino acids and ammonium sulfate) or Spider (1% nutrient broth, 1% mannitol, 0.2% K_2HPO_4) in liquid broth or 1.8% agar-containing plates as described previously (Odds, 1985; Scherer and Magee, 1990; Sherman, 2002). Ura^- auxotrophs were selected on minimal defined medium supplemented with 625 mg 5-fluoroorotic acid and 25 mg uridine per liter (FOA medium). Prior to selection, cells were plated on YPD medium and incubated for 48 hours. Individual colonies were taken from the plate and suspended in sterile distilled water. About ~5×10^6 cells of suspension was spreaded FOA medium and incubated for 2-3 days. Before growing cells in liquid medium, stock cultures were grown on agar plates and stored at 4°C. All cells were grown at 28°C.

1.2. Bacterial strain and culture condition

Escherichia coli DH5α and BL21 (DE3) were used for DNA manipulation and overexpression of proteins. *E.coli* strains were grown 37℃ in Lurea-Bertani (LB, 1% tryptone, 0.5% yeast extract, 1% NaCl) medium supplemented, where required, with the following antibiotic at final concentrations: ampicillin, 50 μg/ml; chloramphenicol, 34 μg/ml.

2. Methods

2.1. Lithium acetate transformation of *C. albicans*

To introduce recombinant construct to disrupt or overexpress, lithium acetate transformation was performed using URA auxotroph, *C. albicans* CAI4 (Gietz *et al.*, 1995) with some modification (Walther and Jürgen, 2003). The *C. albicans* cells were grown overnight from a portion of a single colony in 25mL YPD at 28°C with agitation to a density of 1×10^7 CFU/ml (O.D. > 0.5) and cooled cells on ice and harvested by centrifugation at 9,000 rpm for 5 min at 4℃. Harvested cells were washed twice with 10 mM Tris-HCl, 1.0 mM EDTA buffer (TE buffer, pH 7.5), washed with TE/LiAc buffer (100 mM LiAc, 10 mM Tris-HCl; pH 7.5) and then resuspended pellet (5×10^8 cells) in 100 μl TE/LiAc buffer. Each transformation was added sequentially; 300 μl freshly prepared polyethylene glycol 3550 (PEG 3550, Sigma Aldrich), 50 μl of cells suspension, 6 μg of carrier DNA (Clontech) and 5 μl recombinant DNA as prepared. Transforming cells were incubated for 30 min at 28℃ following heat shock at 42℃ for 15 min. After centrifugation 9,000 rpm for 15 s, supernatant were removed and resuspended pellet spread aliquots on selective medium and incubated at 28℃ for 3 to 5 days.

2.2. Genetic manipulation methods

General techniques for isolation and manipulation of DNA in E. coli were as previously described (Hanahan, 1983; Russel and Holmgren, 1988). pGEM-T easy vector (Promega) was used for cloning of PCR product. Integrating expression vector pQF18 (Feng *et al.*, 1999) and extrachromosomal expression vector YPB1-ADHPt (Csank *et al.*, 1998) were used for introducing appropriate genes into *C. albicans*. The constructs and plasmids used in this study were

summarized in Table 2.

2.3. Polymerase Chain Reaction (PCR)

DNA fragment amplification was performed according to the method recommended by Taq polymerase manufacturer (Promega, Madison, WI) with slight modification. For the reactions, 100 pmol of degenerated oligonucleotide primers, 200 ng of genomic DNA or 10 ng plasmid DNA and 0.5 units of Taq polymerases were combined in a final volume of 50 μl with reaction buffer (50 mM KCl, 1.2 mM $MgCl_2$, 10 mM Tris-HCl, pH 8.4, 0.01% gelatin) containing 50 μM dNTPs. The reaction mixture was subjected to 30 cycles of 1 min denaturation at 94℃, 30 sec annealing at 55℃ and 1 min extension at 72℃.

2.4. RNA extraction and northern blot analysis

Total RNA from cells grown in YPD or Spider broth or agar plate were prepared by the hot phenol extraction method according to the method described by Köhrer and Domdey (1991). A total of 20 μg RNA from each sample was separated on a 1.0% (w/v) agarose gel containing 0.22 M formaldehyde (Sigma-Aldrich) and transferred to a Hybond-N^++ nylon membrane (GE Healthcare) by electrophoresis. The specific probes were prepared by PCR and were labeled with [α-32P]-dATP for 16 h at 30°C. The primer sequences of each probe for hybridization were summarized in Table. Hybridization was performed using various probed dissolved in Rapid-Hyb buffer (GE Healthcare) according to the manufacturer's instructions. The blots were incubated in Rapid-Hyb buffer without the probe for 1 h and then probe was added for 2 h at 65°C. The blot was washed twice with SSC buffer (0.1% SS, 0.3 M NaCl, 30 mM trisodium citrate) for 10 min. The signal was

developed by the bio-imaging system BAS-2500 (FujiFilm). All primers indicated Table 3.

2.5. Quinoxaline derivatives and high pressure liquid chromatography analysis (HPLC)

To determine the intracellular concentrations of α-ketoaldehydes and α-ketocarboxylic acids, the conversion into quinoxaline derivatives was performed using 1,2-diaminobenzene as previously reported, with minor modifications (Cordeiro and Freire, 1996). The harvested cells were disrupted by 2.5 volumes of 0.5 M $HClO_4$, 1,2-diaminobenzene was added to a final concentration of 10 mM, and this mixture was incubated at 37°C for 45 min. All quinoxaline derivatives were purified by a SPE cartridge containing C18 resin based on hydrophobicity (Waters). Quinoxaline derivatives filtered with a 0.22 μm-cellulose acetate membrane were separated by an Agilent 1200 series HPLC system with a Zorbax Eclipse XDB-C18 analytic column (4.6 × 150 mm, Agilent Technologies, USA). The mobile phase was 67% 25 mM ammonium formate buffer, pH 3.4, 3% ACN and 30% methanol for 45 min. A total of 15 μl of each sample of was injected. The flow rate was 0.7 ml/min, and quinoxaline derivatives were detected at a wavelength of 336 nm.

2.6. Measurement of glutathione (GSH) concentration

To determine the cellular glutathione content, extracted cells were reacted with monobromobimane (mBBr) to form derivates and then analyzed as described by Newton and Fahey (1995) with some modification. All cells grew in YPD or Spider medium for 16 hours and were harvested by centrifugation at 9,000 rpm for 5 min at 4℃. Prepared cells were extracted with

Table 1. Strains used in this study

Strain	Genotype	Sources or references
***E. coli* strain**		
DH5α	F⁻ *ΔlacU169(Φ80lacZΔM15)endAlreclhsdR17 deoR supE44 thi-1 λ⁻gyrA96 relA1*	Hanahan (1983)
***C. albicans* strains**		
SC5314	Wild type clinical isolate	Fonzi W.A. and Irwin M.Y. (1993)
CAI4	Δ*ura*3::*imm*434/Δ*ura*3::434	Fonzi W.A. and Irwin M.Y. (1993)
YB103	*Δura3*::*imm434*/*Δura3*::*imm434* *Δglr1*::*hph*/*Δglr1*::*MAL2-GLR1::URA3*	Baek (2003)
YB103Ra	*Δura3*::*imm434*/*Δura3*::*imm434* *Δglr1*::*hisG-URA3-hisG::MAL2-GLR1/GLR1*	This work
YB103Rb	*Δura3*::*imm434*/*Δura3*::*imm434* *Δglr1*::*hisG::MAL2-GLR1/GLR1*	This work
YB103R	*Δura3*::*imm434*/*Δura3*::*imm434* *Δglr1*::*hisG*::*MAL2-GLR1*/*Δglr1*::*GLR1::URA3*	This work
YB104	*Δura3*::*imm434*/*Δura3*::*imm434* (pYPB1-ADHPt)	Baek (2003)
YB105	*Δura3*::*imm434*/*Δura3*::*imm434* (pGLR1-ADHPt)	Baek (2003)
JKC19	*Δura3::imm434/Δura3::imm434* *Δcph1::hisG/Δcph1::hisG-URA3-hisG*	Liu *et al.* (1994)
HLC52	*Δura3::imm434/Δura3::imm434* *Δefg1::hisG/Δefg1::hisG-URA3-hisG*	Lo H.-J. *et al.* (1997)

BCa2-10	*Δura3::imm434/Δura3::imm434 Δtup1::hisG/Δtup1::hisG-URA3-hisG*	Braun B.R. and Johnson A.D. (1997)
HLC54	*Δura3::imm434/Δura3::imm434 Δcph1::hisG/Δcph1::hisG Δefg1::hisG/Δefg1::hisG-URA3-hisG*	Lo H.-J. *et al.* (1997)

Table 2. Plasmids used in this study

Plasmid	Descriptions	Sources or references
pGEM-T easy	PCR cloning vector; Ap^r	Promega
pYPB1-ADHPt	Expression vector of *Candida albicans*	Csank *et al.* (1998)
p5912	*hisG-URA3-hisG* cassette in pUC18	Fonzi W.A. and Irwin M.Y. (1993)
pQF18	*hph-URA3-hph* cassette in pBluescript KS	Feng *et al.* (1999)
pQF181	pGEM-T easy vector containing *hph*-URA3-*hph* cassette which can be excised with *Sac*I/*Hind*III (Forward)	Hwang *et al.* (2003)
pQF182	pGEM-T easy vector containing *hph*-URA3-*hph* cassette which can be excised with *Sac*I/*Hind*III (Reverse)	Hwang *et al.* (2003)
pYB101	*GLR1* deletion construct with YB1a-YB1b/YB1c-YB1d fragment containing*hph-URA3-hph*	Baek (2003)
pYB102	*GLR1* deletion construct with *CIp10-MAL2p-GLR1-URA3*	Baek (2003)
pYB103	*GLR1* deletion construct with *CIp10-MAL2p-GLR1-URA3* in p5921	This work
pGLR1-ADHPt	pYPB1-ADHPt containing *GLR1* ORF	Baek (2003)
pFBA1-ADHPt	pYPB1-ADHPt containing *FBA1* ORF	Ku (2014)
pTDH3-ADHPt	pYPB1-ADHPt containing *TDH3* ORF	This work

50% aqueous acetonitrile (Sigma-Aldrich) containing 40 mM HEPES (pH 8.0) and 2 mM mBBr (Sigma-Aldrich). After incubation at 60℃ for 15 min, the samples were acidified with 5 μl of 5 N methanesulfonic acid (Sigma-Aldrich). Resulting supernatant from the crude extract was collected and analyzed using HPLC. Control samples were treated with 10 mM *N*-ethylmaleimide (NEM, Sigma-Aldrich) and incubated at 50℃ for 10 min before derivatization to prevent labeling of thiol group from with mBBr. The concentration of total GSH was determined using 2 mM dithiothretol (DTT), which reduces GSSG to GSH. Samples were passed through a ZORBAX SB-C18 column (4.6 × 250 mm, Agilent Technologies, USA). HPLC was performed using a Water system equipped with a Hewlett-Packard 1050 series fluorescence detector. The mBBr-derived thiol compounds were detected using excitation and emission at 370 and 480 nm, respectively. The mobile phase consisted of buffer A (methanol, HPLC grade from Sigma-Aldrich) and buffer B (0.1% trifluoroacetic acid from Sigma-Aldrich). The proportion of buffer A in the continuous gradients was as follows; 15% at 0-2 min, 25% at 30 min, 100% at 34 min, 15% at 37 min, and 15% at 40 min. If necessary, samples were co-injected with GSH (Duchefa) standards.

2.7. Oxidant resistance assay and cell growth on various thiol compounds

All experiments regarding the susceptibility to various oxidative stresses were performed as proposed previously (Izawa *et al.*, 1995). Cells were grown to mid-logarithmic phase (2×10^7 cells/ml), harvested, resuspended in 0.1 M potassium phosphate buffer (pH 7.0) and were spotted onto SD plates containing various concentrations of oxidants for 3 days at 28°C. Additionally,

to verify the resistance against several types of oxidants, including H_2O_2, menadione, and diamide, *Candida* cells on hyphae-inducing medium treated with appropriate amounts of oxidants were cultured for 4 days at 37℃ as described previously (Kwak *et al.*, 2015).

2.8. Measurement of intracellular reactive oxygen species (ROS) concentration

All strains were grown in YPD or Spider medium at 28 or 37℃. 2 x 10^7 cells were collected from each strain by diluting into 25 mM Tris-HCl, pH 8.5. The cells were washed twice and suspended with ice cold PBS. 20 μM dichloroflurescin diacetate, DCFH-DA (Sigma-Aldrich), was added to prepared cells to measure intracellular ROS content according to the method described by Shen and Scaiano (2006). To examine, a Cary Eclipse Fluorescence Spectrophotometer (Varian) was used. ROS was detected using excitation and emission at 492 and 524 nm, respectively.

2.9. Real-time RT-PCR

All strains were grown overnight and were diluted to an A_{600} of 0.1 in pre-warmed YPD or Spider medium and grown at 28 or 37℃. Cells were harvested from triplicate samples at an appropriate phase. Cells were lysed with a bead beater homogenizer (BioSpec). RNA was isolated from a cell lysate with an RNAiso Plus (TAKARA) reagent following the manufacture's manual. The 5 μg of RNA was used to generate cDNA by SuperScript® III Reverse Transcriptase following the manufacture's manual. The qPCR was accomplished using SYBR premix EX TaqTM (TAKARA) and 7300 Real-Time PCR systems (Applied Biosystems). *ACT1* was used as an internal

Table 3. Primers used in this study

Primer	Sequences
FBA1	5’-ATGGCTCCTCCAGCAGTTTTAAGTAAATC-3’
	5’-GATATTTTCCACACCAAAGGACAATTGT-3’
GLR1	5’-ATGTTTACTAATAGTATAATATCTAAA-3’
	5’-CTAAGTCATTGTGACCAATTCTTCAGC-3’
TDH1	5’-ATGGCTATTAAAATTGGTATTAAC-3’
	5’-TCAAGCAGAAGCTTTAGCAACGTG-3’
TPI1	5’-ATGGCTCGTCAATTTTTCGTAGGTGGC-3’
	5’-TTATAATCTAGATTTGATGATATCAAC-3’
ECE1[a]	5'-TCTCAAGCTGCCATCCA-3'
	5'-AGATTCAGCTGATCTAGTAA-3'
HWP1[a]	5'-CACAGGTAGACGGTCAAGGT-3'
	5'-GATCCAGAAGTAACTGGAACAGAACTT-3'
HYR1[a]	5'-CTTCACCACTGTTCCATGCTCC-3'
	5'-GCTCCTGAACCATTTCCTGAACC-3'
ALS1[a]	5'-CAGATGCTTCAACAATTTACATTG-3'
	5'-TCACTAAATGAACAAGGACAATA-3'
INT1[a]	5'-CCCAAAAAAGATAAAATAAA-3'
	5'-AAATCCAACTGTGGACAAAG -3'
EFG1[a]	5'-TCAACGTATTCTATACCCTATTAC-3'
	5'-CTTTTCTTCTTTGGCAACAGTGCT-3'
CPH[a]	5'-TCAATTACTAAAACATACAATGGTGATCC-3'
	5'-CGTGGCAGCAGCAGTAGTAGCAACTGCAC-3'
TUP1[a]	5'-CGCGGATCCCCACCAGCAATGTCCATGTA-3'

	5'-GCGGGTACCGCGATGTTGACGGGTGCTGT-3'
ACT1[b]	5'-CAGACCAGCTGATTTAGGTTTGG-3'
	5'-CCGTATAATTCTTTTCTAACATCCATGT-3'
FBA1[b]	5'-TGAATACTTGAAAGCACCAGTTGGT-3'
	5'-TTTTCACCTTCTCTAACCCAGACTCT-3'
TDH3[b]	5'-TGAAGAAGGTTTGATGACCACTGT-3'
	5'-TCCAGTCCTTGTGGGATGGA-3'
ADH1[b]	5'-CACGATGGTTCATTCGAACAA-3'
	5'-GGTGCGACATTGGCTAAATCA-3'

* Sources are all from this work.
[a] used in Northern blot analysis.
[b] used in real-time RT-PCR.

standard. All primers indicated Table 3.

2.10. Western blot analysis

SDS-PAGE was performed on 12% polyacrylamide-denaturing gel. Electro-transfer of proteins from the gel to nitrocellulose membrane (Protran BA83, Schleicher & Schuell) was carried out as previously described (Towbin *et al.*, 1992). Nonspecific space on the transferred membrane was blocked by Tris-buffered saline (TBS; 10 mM Tris-HCl, pH 7.5 and 150 mM NaCl) containing 5% Skim milk, 0.02% sodium azide for 15 min at 25℃ three times. The membrane was then incubated in TBST (10 mM Tris-HCl, pH 7.5, 150 mM NaCl and 0.05% Tween20) with primary antibody for overnight at 4℃. After washing twice with TBST for 15 min, the membrane was incubated for 1 hour at 4℃ with secondary antibody solution (anti-mouse IgG antibody-alkaline phosphatase, diluted to 1:10000 in TBST). The membrane was then washed for 15 min with three changes of TBST and rinsed with alkaline phosphatase buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl and 10 mM $MgCl_2$). The TBST washed membrane was developed in 20 ml of alkaline phosphatase buffer containing BCIP (50 mg/mL) and NBT (75 mg/mL) at 25℃.

2.11. Glutathione reductase activity assay

The activity assay of glutathione reductase was measured spectrophotometrically in 10 mM potassium phosphate, pH 7.4, 1.0 mM EDTA, 20 mM GSSG, 2.0 mM NADPH and an aliquot of crude extracts (Baek, 2003). The decrease of the absorbance at 340 nm due to the oxidation of NADPH (ε_{340} = 6,220 $M^{-1}cm^{-1}$) was monitored during the first 1 min of the reaction at 30℃.

One unit (U) of the enzyme was defined as the amount of enzyme that consumed 1 μmol NADPH/minLigand fishing experiment.

2.12. Statistical analysis

The results are presented as the means ± standard deviation (SD). The statistical significance of the differences was tested using Student's t-test in Microsoft Office Excel (2015). For all comparisons, values of $p < 0.05$ (*) were considered statistically significant.

III. Results and Discussions

1. *GLR1* characteristics and its importance for cell growth in budding *C. albicans*

To elucidate the physiological roles of *C. albicans GLR1*, a conditional disruptant YB103, and its revertant YB103R were obtained by sequential disruption (Baek, 2003). Noticeably, Baek (2003) repeatedly failed to disrupt the *GLR1* gene using *C. albicans* conventional URA-blast method (Fonzi and Irwin, 1993) through several types of disruption cassette-containing plasmids, including the p5921 (p5922) (Kwak *et al.*, 2015) and pQF181 (pQF182) (Hwang *et al.*, 2003). When proven to use both the *hisG-URA3-hisG* and *hph-URA3-hph* cassettes to disrupt *GLR1*, the homologous recombination did not properly take place in disruption stages. Moreover, the *GLR1* disruptant could not be constructed fundamentally based on the stringent triple auxotrophic strain, BWP17 (*ura3Δ::imm434/ Δura3Δ::imm434, his1Δ::hisG/ his1Δ::hisG, arg4Δ::hisG/arg4Δ::hisG*) because it was readily demonstrated that arginine-induced germ tube formation in *C. albicans* was strongly stressed in previous experiments upon the interaction between *Candida* pathogenicity and murine macrophage line RAW 264.7 (Ghosh *et al.*, 2009) or *Fusobacterium nucleatum* (Wu *et al.*, 2015). The arginine metabolism via arginase (Car1p) and urea amidolyase (Dur1,2p) significantly contributes to hyphal switching and provides a central escape mechanism from the host defense (Ghosh *et al.*, 2009). Furthermore, the interaction between the macrophage line RAW 264.7 and *C. albicans* was occurred by endogenous or exogenous arginine density-dependent manner followed by activating an Efg1p-dependent yeast-to-hypha dimorphic conversion, enabling wild-type *Candida* cells to escape from macrophages (Ghosh *et al.*, 2009). Thus, two mutants were unable to synthesize arginine,

BWP17 or SN152, and were defective in making hyphae inside the macrophages despite the germ tube formation of arginine prototrophs susceptible to a pathogenicity-related function (Ghosh *et al.*, 2009). This experimental evidence coincided with the result that the firm coaggregation between wild-type *F. nucleatum* 23726 and *C. albicans* SN152 mutant in an *in vitro* assay could be remarkably inhibited by arginine and mannose supplementation (Wu *et al.*, 2015). This investigation also indicates that arginine metabolism by *C. albicans* BWP17 strain might affect hyphal formation essentially required for host defense thereby result in greatly experimental elusiveness to evaluate *Candida* full virulence and hyphal formation presumably affected by the BWP17-derived *GLR1* disruption in this study.

Additionally, *C. albicans* strains SN87, SN95, and SN152, respectively, bearing the homozygous null mutations, including *HIS1* and *LEU2*, *HIS1* and *ARG4*, and *HIS1*, *LEU2*, and *ARG4* (*leu2Δ/leu2Δ*, *his1Δ/his1Δ*; *arg4Δ/arg4Δ*, *his1Δ/his1Δ*; and *arg4Δ/arg4Δ*, *leu2Δ/leu2Δ*, *his1Δ/his1Δ*) (Noble and Johnson, 2005) addressed the minimal effect on *C. albicans* virulence in a mouse model similar to that of wild-type SC5314. However, the possible differences in the hyphal growth and other metabolic alterations exerted by these auxotrophic strains were not elucidated. On the basis of these critical experimental evidences of BWP17 (Ghosh *et al.*, 2009; Wu *et al.*, 2015) and other auxotrophic strains (Noble and Johnson, 2005), Baek (2003) constructed disruptants of *GLR1* by placing the remaining *GLR1* allele under the control of *CaMAL2* promoter as suggested (Backen *et al.*, 2000) to avoid any experimental false driven by genetic manipulation of *C. albicans GLR1* and to

address the exact examination of morphological switch or virulence and metabolic variations predicted by Glr1 actions, not by GSH alone.

Compared to SC5314 and YB103R, the *GLR1*-deficient YB103 showed the severe growth defect when *GLR1* expression under the control of the *CaMAL2* promoter was conditionally repressed by glucose (Figs. 1 and 2), indicating that *GLR1* gene is required for cell growth. This result was consistent with the disruption strategy using *CaMAL2* promoter of *C. albicans DPB2*, encoding potential DNA polymerase epsilon subunit, to demonstrate that the gene is essential and its loss leads to a hypha-like morphology coincident with ceasing proliferation (Backen *et al.*, 2000). However, YB103 cells grown in SD agar plates and liquid media with supplements, such as GSH, GSSG, *N*-acetylcysteine, cysteine, and dithiothreitol, generally underwent partial rescue of cell growth (Scheme 3A and fig. 3). Astonishingly, the growth rate of YB103 could not be fully restored by exogenous GSH (Scheme 4A) contrast to the GSH-supplemented *GCS1*-deficient cells, which showed the near-normal growth during GSH replenishment (Baek *et al.*, 2004; Kwak M.-K. *et al.*, 2014). Additionally, *C. albicans glr1Δ* cells were previously observed to have growth defects even in YPD medium (Tillmann *et al.*, 2015). These experimental clues strongly suggest that *Candida GLR1* expression might crucially function as a cell fate decision factor including GSH reduction capacity and antioxidative defenses, seemingly despite the presence of *GCS1* gene expressions for GSH biosynthesis (Baek *et al.*, 2004). This result also provides that the *GLR1* is an essential requirement for normal growth rather than GSH itself, and its parallel system such as Trx or Grx may not function properly.

Although the Trx and Grx are ubiquitous in eukaryotes and their genes can be searched using genome database (Jones *et al.*, 2004), *C. albicans* gene sets

related to various induced stresses did not always corresponding stresses in the case of Trx and Grx enzymatic systems (Enjalbert *et al.*, 2003). Interestingly, when the oxidized thiol, GSSG, was exogenously added, partial rescue of growth rate occurred in YB103 cells (Scheme 4A), partly coinciding with the result that an increased cellular GSSG, which is toxic, could trigger other enzyme systems partly independent of GR in *E. coli* (Miranda-Vizuete *et al.*, 1996; Russel and Holmgren, 1988; Tuggle and Fuchs, 1985). The growth of GSH auxotroph mutants can be rescued by exogenous thiol compounds other than GSH, suggesting low GSH/GSSG ratio (or high GSSG) might be responsible for growth defects and alternative systems that can reduce GSSG in *C. albicans*. Therefore, partially recovered growth of *C. albicans* with exogenous GSSG in Scheme 4A may be able to explain with other growth-recovery systems, not with Trx and Grx in *C. albicans*. Nevertheless, it seems possible that GSSG-scavenging functions might be engaged by some types of redox systems, which can enhance transcript levels of sets of genes, including putative thiol peroxidases (*TSA1*, *PRX1*, *AHP11*, and *AHP12*) and GSH-transferase putative genes (*GTT12*, *GTT13*, *GTT14*, and *GST3*), when given of oxidative stresses in *C. albicans* (Michán and Pueyo, 2009). However, because there is no reference to evaluate the GSSG effect on the cell growth recovery when using maltose as C-source in the case of *GLR1*-deficient YB103 strain, this phenomenon's mechanism based on our experimental evidence remains unclear.

Considering the severe growth defects by *GLR1* disruption despite the GSH supplementation exogenously, we hypothesized that cellular GSH itself might not fully contribute to *C. albicans* cell growth. As expected, although cellular GSH was decreased in YB103 cells, endogenous GSH remained (Fig. 4),

indicating that *GLR1* expression crucially affects cell viability, independent of endogenous- or exogenous GSH. This result was completely contrasted to that previously reported for diminished cellular GSH concentrations in GSH-depleted and GSH-supplemented *gcs1* disruptant in *Dictyostelium* (Kim *et al.*, 2014). Thus, GSH deficiency in YB103 cells compared to reference strains did not seem to be the leading cause of severe growth defect of this strain, coinciding with the previous experiment of incomplete growth recovery in YB103 even in the presence of exogenous GSH (Scheme 4A). Based on significantly lowered GSH levels in this strain, to address the absolute requirement of *GLR1* affecting cell growth, I examined the sensitive oxidative damages reflected by cellular MG and ROS accumulation in this strain. The significantly higher cellular contents of these metabolites were displayed compared to reference experiments when quantified (Fig. 5). The YB103 cells displayed significantly accumulated cellular ROS and MG similarly as seen by the GSH-depleted *Candida* or *Dictyostelium* cells (Choi *et al.*, 2008; Kwak *et al.*, 2014; Kwak *et al.*, 2016) and exhibited filamentous morphology (Fig. 6). Therefore, *Candida GLR1* as a cell fate regulator seems to participate in the GSH pool to maintain cellular redox state even in the possible presence of other redox enzymes irrelevant of the existence or absence of GSH.

2. The resistance against oxidants or macrophage attack by non-hyphal forming *GLR1*-overexpressing cells

When observing the Glr1 activity in budding *GLR1* mutants, the *GLR1*-deficient and -overexpressing YB103 and YB105 cells grown in SM and SD broth showed up to 19.16- and 18.58-fold decreased and increased Glr1 activity

spectrophotometrically, respectively (Baek, 2003). Also, the remarkably high GR activity of YB105 cells displayed significantly increased resistance to various stresses (Scheme 4B). My laboratory members previously observed that GR activity was enhanced in *sod2/sod2 C. albicans*, which is more sensitive to different oxidants than wild-type. I expected that *GLR1* overexpression would render *C. albicans* resistant to diamide toxicity because the *sod1*- and *sod2*-null mutants were known to be resistant to diamide, possibly due to increased GR activity (Hwang *et al.*, 2003). Importantly, whether the *GLR1* expression is induced by various types of stresses compared to other microbes or not, Baek (2003) performed western blot analysis with wild-type SC5314 crude extracts treated with several external stresses. Herein, 50 μM menadione, 1 mM H_2O_2, and 0.5 mM diamide, which oxidize free thiol groups, significantly induced the *GLR1* expression. Additionally, to test whether the expression of *GLR1* is affected by other stresses, Baek (2003) subjected cells to various conditions including high salt concentrations, which are known to cause oxidative stress along with osmotic stress. In the case of *S. cerevisiae*, high temperature (Piper, 1995) and ethanol (Costa *et al.*, 1997) treatment also generate ROS, yielding oxidative stresses.

However, only treatment with 100% O_2 increased *GLR1* expression (Baek, 2003) and the other treatments did not change the expression level of *GLR1*, while *pgr1+* encoding GR from *S. pombe* was induced by these stresses (Lee *et al.*, 1997). Meaningfully, these results suggest that the response to the stresses tested here might be different in the two yeast types and that *Candida* Glr1 is commonly involved in response to oxidative stresses. Simultaneously, the exogenous stresses used in the resistance tests induced *GLR1* expression (Scheme 4B), demonstrating that *GLR1* overexpression confers more resistance

against various stresses on the *C. albicans* cells fundamentally due to the significant higher Glr1 activity (Fig. 7). *S. pombe* strains with overexpression of GR showed similar results, except menadione treatment (Lee *et al.*, 1997). This difference may be due to a different response mechanism to redox cycling agents, which is known to generate superoxide. Phagocytic cells generate ROS to defend host cells from microbial infection. To circumvent this environment, pathogenic microbes require efficient antioxidant systems. In the case of *C. albicans*, null mutants of antioxidant proteins such as *SOD1* (Hwang *et al.*, 2002) or *CAT1* (Wysong *et al.*, 1998) are more susceptible to the fungicidal activity of phagocytic cells than wild-type and revertant cells. Therefore, to demonstrate whether the antioxidant activity of Glr1 protects *C. albicans* from the fungicidal action of macrophages, Baek (2003) measured the survival rate of the *GLR1*-overexpressing YB105 in the cell line RAW264.7. When cultured in the presence of macrophages, the YB105 cells exhibited slightly increased colony formation (24.60 ± 2.8%) compared to the reference strain (20.45 ± 2.15%). These results suggested that *GLR1* overexpression rendered *C. albicans* resistant to various stresses and phagocytic macrophage engulfment. Thus, the increased Glr1 by *GLR1* overexpression also enhanced resistance against not only oxidative stresses but also phagocytosis by macrophages.

The experimental evidence of highly maintained Glr1 activity in budding YB105 cells (Fig. 7) compatible with resistance against various oxidants (Scheme 4B) again prompted us to measure the Glr1 activity and resistance in hyphal-inducing medium (Figs. 7 and 8). The Glr1 activity from Spider media was shown to be 17.9- and 17.2-fold higher than SC5314 and YB104 cells, respectively. This experiment gave similar results with the previous measurement of Glr1 activity in budding YB105 cells (Fig. 7). Surprisingly, the

GLR1-overexpressing YB105 strain showed a completely defective hyphal growth in Spider solid media (Fig. 8). This budding yeast form nearly maintained during treatment of several oxidants. Non-hyphal phenotype by *GLR1* overexpression was fully different from those of previous reports that exogenous GSH did not contribute to reducing hyphal formation in wild-type SC5314 when using YNB, YNB-serum, and Spider agar medium (Guedouari *et al.*, 2014). Moreover, the ability of a fungal cell associated with environmental stresses and host defenses was strongly influenced by its metabolic state based on GSH-dependent glutathionylation (Gergondey *et al.*, 2016). Thus, while mainly focused on GSH and its affecting metabolism by the GSH action itself in wild-type SC5314 (Gergondey *et al.*, 2016; Guedouari *et al.*, 2014), the effect of YB105 entirely maintained by *GLR1* expression, not by GSH actions (Figs. 7 and 8; Baek, 2003).

3. Non-hyphal growth and attenuate virulence by GLR1 overexpression

The data regarding both the resistance against oxidants and enhancement of phagocytosis by macrophages of YB105 cells support my hypothesis that the increased cellular Glr1 activity by *GLR1* expression promote redox homeostasis and *C. albicans*-phagocyte interactions during infection (Figs. 7, 8 and 9; Baek, 2003). These phenomena coincided with the previous work that the *Glr1* and *Fdh3* mutants based on both BWP17-derived disruption and *tetON* promoter-using doxycycline-dependent overexpression, respectively, which were genetically different from our *GLR1* mutants, gave similar roles during the adaptation of *C. albicans* cells to oxidative stresses and macrophage killing (Tillmann *et al.*, 2015). Considering their effects during fungus-phagocyte

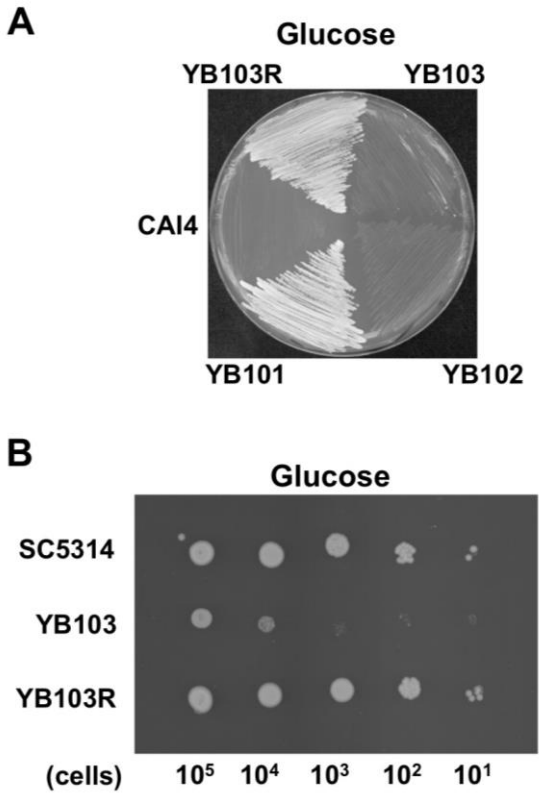


Fig. 1 Conditional repression of *GLR1* gene under the control of *CaMAL2* promoter in *C. albicans.* (A) Each strain of CAI4 (*GLR1/GLR1*), YB101 (*glr1::hph-URA3-hph/GLR1*), YB102 (*glr1::hph/GLR1*), YB103 (*glr1/glr1::MAL2-GLR1::URA3*) and YB103R (*GLR1::MAL2-GLR1::URA3*) was streaked on minimal medium containing glucose. Plates were incubated for 3 days at 28°C. (B) Each strain was grown to saturation and spotted in serial of 1/10 dilution on minimally defined SD containing glucose. Plates were incubated for 3 days at 28°C.

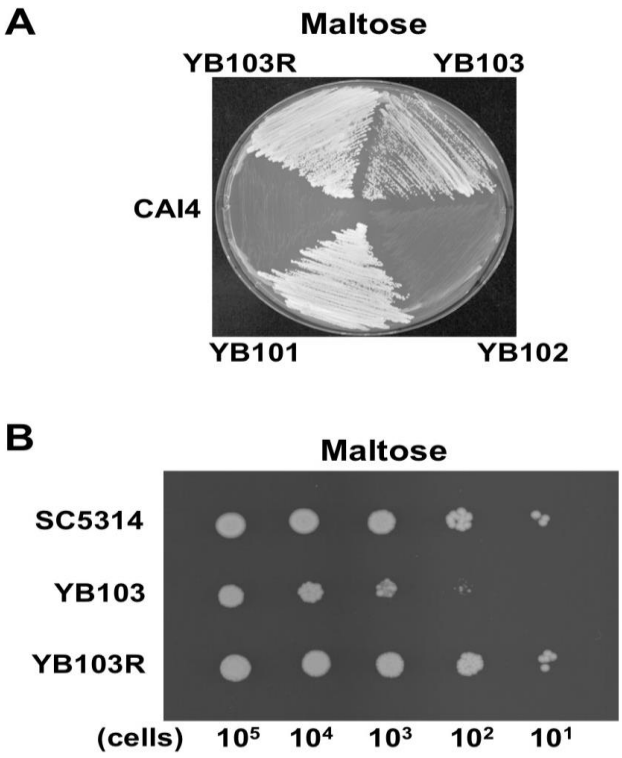


Fig. 2 Conditional repression of *GLR1* gene under the control of *CaMAL2* promoter in *C. albicans*. (A) Each strain of CAI4 (*GLR1/GLR1*), YB101 (*glr1::hph-URA3-hph/GLR1*), YB102 (*glr1::hph/GLR1*), YB103 (*glr1/glr1::MAL2-GLR1::URA3*) and YB103R (*GLR1::MAL2-GLR1::URA3*) was streaked on minimal medium containing maltose. Plates were incubated for 3 days at 28°C. (B) Each strain was grown to saturation and spotted in serial 1/10 dilution on SM medium containing maltose, respectively. Plates were incubated for 3 days at 28°C.

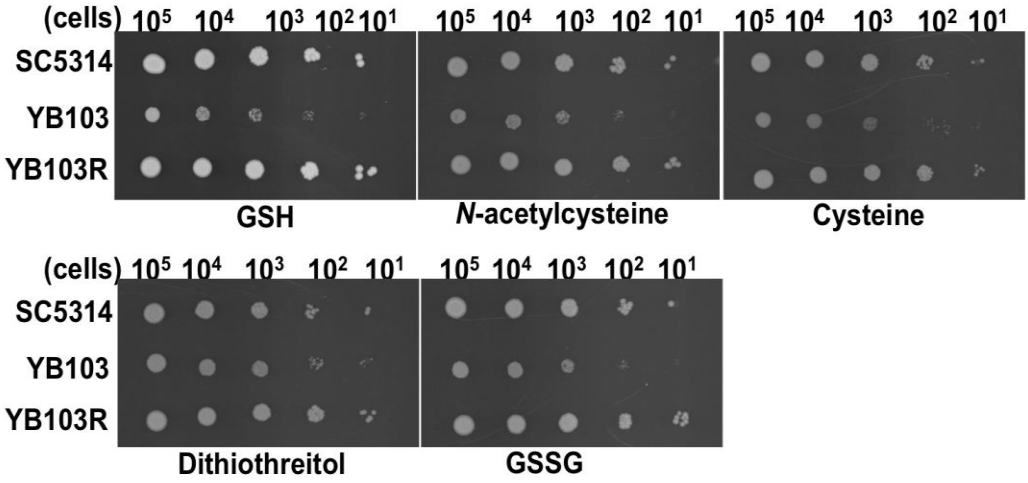


Fig. 3 The effect of thiols on the growth of *GLR1*-deficient mutants. Each strain of SC5314 (*GLR1/GLR1*), YB103 (*glr1/glr1::MAL2-GLR1::URA3*) and YB103R (*GLR1::MAL2-GLR1::URA3*) was grown to saturation and spotted as serial 1/10 dilutions on minimal medium containing glucose supplemented with various thiols (each at 1 mM). Plates were incubated for 3 days at 28°C.

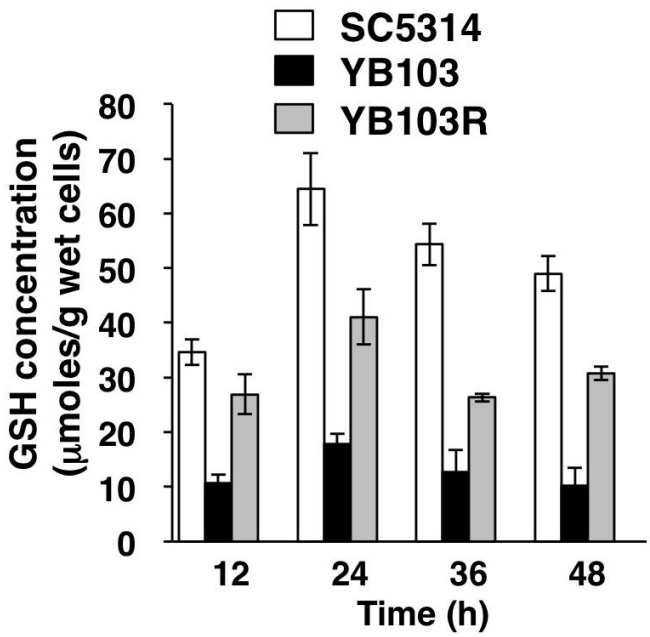


Fig. 4 The cellular concentrations of GSH in *GLR1*-deficient mutants. Each strain of SC5314 (*GLR1/GLR1*), YB103 (*glr1/glr1::MAL2-GLR1::URA3*) and YB103R (*GLR1::MAL2-GLR1::URA3*) was grown to saturation. The cellular GSH content was measured using analytic HPLC as described in Material and Methods section.

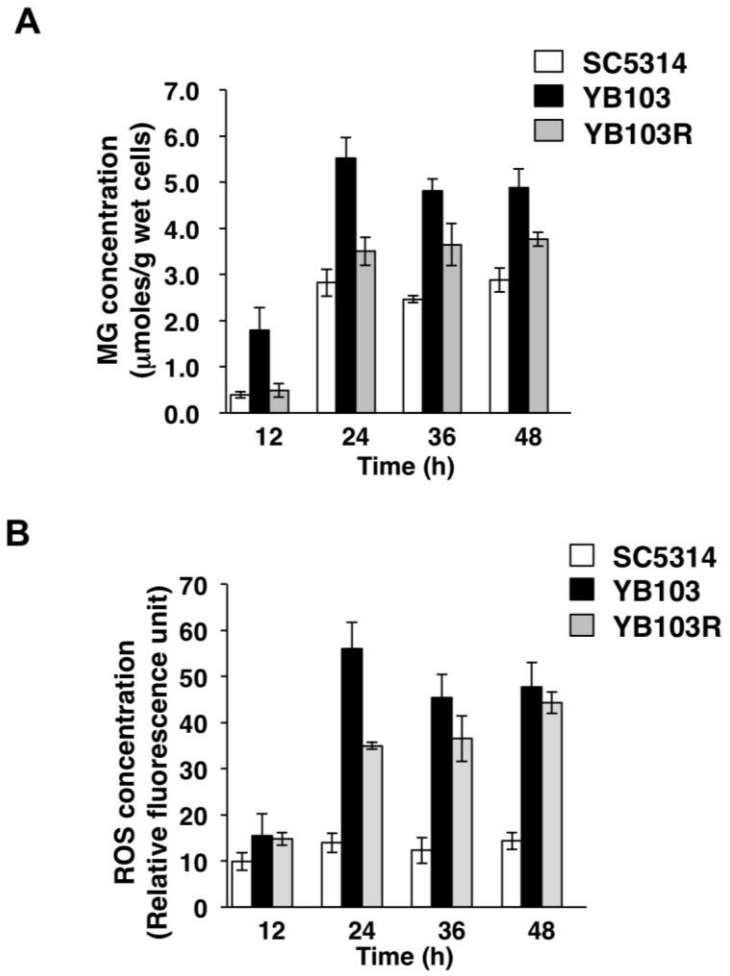


Fig. 5 The cellular concentrations of MG and ROS in *GLR1*-deficient mutants. Each strain of SC5314 (*GLR1/GLR1*), YB103 (*glr1/glr1::MAL2-GLR1::URA3*) and YB103R (*GLR1::MAL2-GLR1::URA3*) was grown to saturation in minimal medium containing maltose. (A) The intracellular MG levels in YB103R by quinoxaline derivative quantifications and (B) the relative ROS concentrations using a fluorescence spectrometry were shown. The values represent the averages ± standard deviation of three independent experiments.

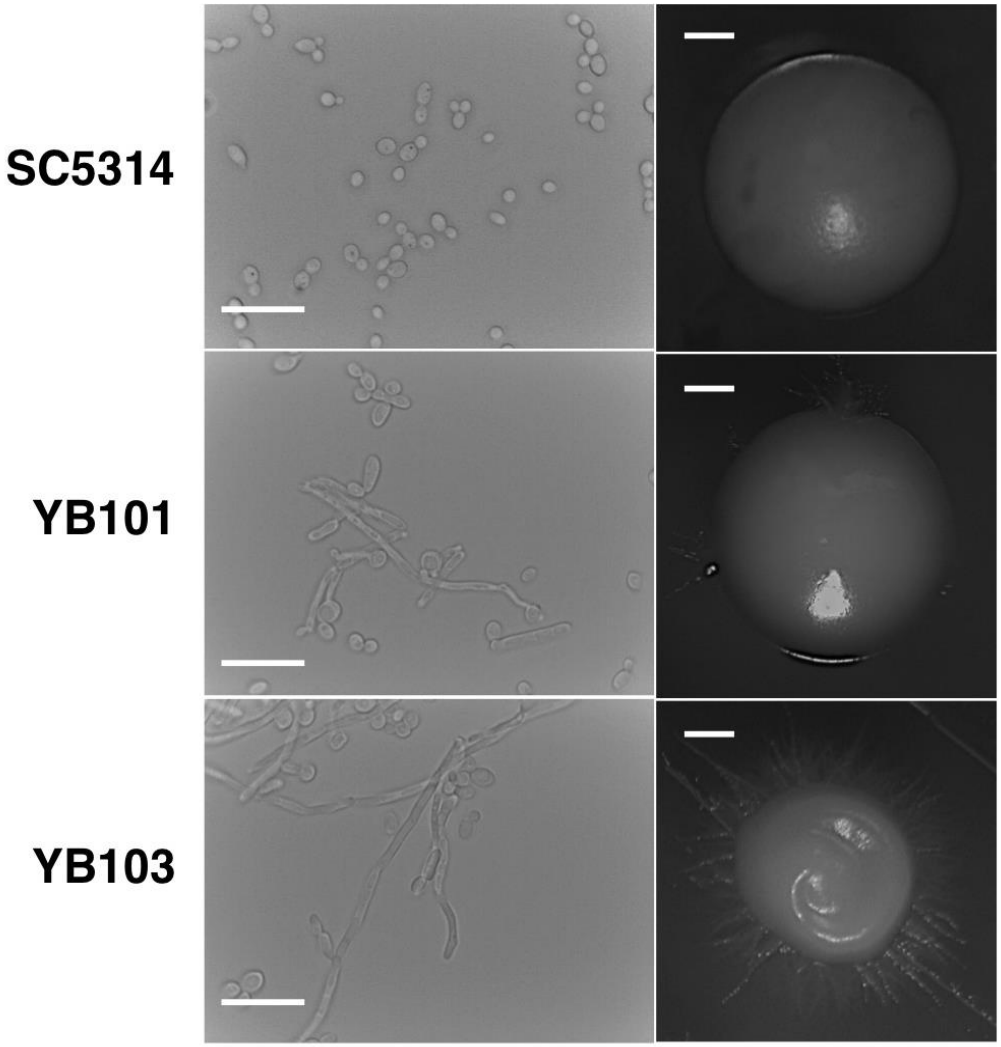


Fig. 6 *GLR1* deficiency leads to hyphal growth in *C. albicans*. Each strain of SC5314 (*GLR1/GLR1*), YB101 (*GLR1/glr1*), YB103 (*glr1/glr1::MAL2-GLR1::URA3*) was exponentially grown in YPD medium at 28°C. Each cell was scraped from a colony. Scale bar for cells: 20 μm; Scale bar for a colony: 0.5 mm.

interactions by showing non-hyphal formation in hyphal-inducing solid media (Fig. 9), *GLR1* overexpression cells exhibited the slight filamentation, and its cellular GSH decreased in a time-dependent manner in 10% serum and Spider liquid media (Fig. 10A; Baek, 2003). It was suggested that non-hyphal formation of YB105 (Fig. 9) even under the oxidative stresses seemed to be correlated with cellular MG content, strongly due to the MG accumulation by GSH deficiency as proposed previously (Kwak *et al.*, 2014; Kwak *et al.*, 2015). As expected, cellular MG in Spider liquid media was increased reversely proportional to GSH content in YB105 strain (Fig. 10A), indicating that the reciprocal changes in these metabolites might be involved in *Candida* dimorphism.

To investigate the effect of *GLR1* overexpression on hyphal growth in the absence of oxidative stresses, YB105 cells were observed when grown on solid Spider, Corn meal agar, and SLAD medium (Fig. 11). Herein, the hyphal formation was not seen in the YB105 strain, identical to budding yeast, while the reference strain had formed extensive agar-invasive hyphae by 5 days (Fig. 11A). Thus, cellular GSH content by *GLR1* expressions might be assumed to alter significantly in YB105 cells grown in hyphal-inducing media (Figs. 4, 5 and 10). This assumption was experimentally elucidated in next section. To confirm the morphological differences caused by *GLR1* overexpression, the mRNA levels of hyphal-specific genes were analyzed (Fig. 11B). *ECE1* (Birse *et al.*, 1993) and *HYR1* (Bailey *et al.*, 1996) are known to be specifically expressed during hyphal growth in *C. albicans* and do not affect virulence. These hyphal specific mRNAs were expressed in YB104 grown in Spider media at 37℃, while the YB105 cells were suppressed in the expression of *ECE1* and *HYR1*, consistent with the morphological data. This result indicated

that the *GLR1* seemed to be involved in the hyphal growth as a negative regulator, which entirely associated with *C. albicans* viability drove by cellular GSH-dependent redox cycling balance commonly in both budding yeasts and hyphae (Guedouari *et al.*, 2014).

Additionally, as illustrated in Baek (2003), all the mice injected with SC5314 and YB104 died within 11 days after injection. In contrast, 50% of the mice injected with the *GLR1*-overexpressing strain YB105 survived to the end of the experiment. A Kaplan-Meier log rank test revealed that the survival differences among the strains were statistically significant ($P < 0.0001$), with an extended median survival time of 29 days for the YB105 injection compared to 6.5 and 8.5 days when injected with SC5314 and YB104, respectively. These infection data are fully consistent with the macrophage killing effect and show that GLR1 contributed to the *Candida* full virulence. Fig. 12 represents the effect of *GLR1* overexpression on the extent of transcription level of virulence factors of *C. albicans* cultured in Spider medium at 37℃. YB105 cells exhibited reduced expression of *ALS1* (Fu *et al.*, 2002), *HWP1* (Tsuchimori *et al.*, 2000), and *INT1* (Gale *et al.*, 1998). These results proposed that *GLR1* overexpression attenuates the virulence of *C. albicans* by suppressing the expression of proteins involved in filamentation and adherence. Disruption of *INT1*, a surface protein, also leads to poor hyphal growth depending on culture media (Gale *et al.*, 1998), and the *phr1/phr1* mutant, which lacks a surface protein, exhibits pH-conditional morphological defects (Saporito-Irwin *et al.*, 1995).

Additionally, pathogenicity of *C. albicans* is believed to be closely linked to morphological dimorphism because *C. albicans* strains locked in either the yeast (Lo *et al.*, 1997) or filamentous form (Braun and Johnson, 1997) have in

common avirulent in mice. Adherence to epithelial cells is also an important determinant for the pathogenesis of *C. albicans* (Sundstrom, 2002). Interestingly, the *GLR1* overexpression impaired *C. albicans* virulence in a mouse model. So far, disruption of any antioxidant enzymes has been shown to be attenuated for virulence or have no effect on virulence. Strains lacking *ALO1* (Huh *et al.*, 2001), *SOD1* (Hwang *et al.*, 2002), *CAT1* (Wysong *et al.*, 1998), *ADH1* (Kwak *et al.*, 2014) and *EAPX1* (Kwak *et al.*, 2015) show reduced virulence significantly, while disruption of *SOD2* do not have significant effects on virulence (Hwang *et al.*, 2003). Furthermore, the *GLR1*-overexpressing cells were resistant to oxidative stresses. However, the nonvirulent *cla4/cla4* mutant is more resistant to macrophage than wild-type strain, suggesting that the sensitivity of macrophage cells is not a unique determinant of virulence (Marcil *et al.*, 2002). As mentioned, adhesion is another important factor in the pathogenicity of *C. albicans*, and overexpression of *GLR1* down-regulated expressions of *ALS1* and *INT1* (Fig. 12). These regulatory factors not only affected virulence but also were involved in adhesion to epithelial cells, indicating that *GLR1* overexpression in *C. albicans* resulted in impaired virulence by regulating adhesion. Additionally, in other previous experiments of the virulence test including *G. mellonella* and a three-day murine intravenous challenge infection model, *GLR1* and *FDH3* overexpressing cells were induced by doxycycline (Tillmann *et al.*, 2015). The results were shown to be similar to that in our one-month follow-up study using a mouse infection model (Fig. 12).

4. The Efg1-mediated signaling pathway involved in the non-hyphal phenotype triggered by *GLR1* expression

For reasons that are not clear, *GLR1* overexpression remarkably reduced *Candida* virulence and filamentation. Whatever the molecular basis for the observation, it is clear that the *GLR1* functionality contributed to the *Candida* virulence during systemic infection (Fig. 12). For this reason, I verified whether *GLR1* expression might affect *C. albicans* regulatory signaling pathways including a Cph1-mediated mitogen-activated protein kinase (Liu *et al.*, 1994) and an Efg1-mediated cyclic AMP/protein kinase A pathways (Ernst J.F., 2000) on solid media. GLR1 overexpression led to non-hyphal growth, and cellular GSH content was not always compatible with GLR1 activities in both budding yeast and hyphae grown (Figs. 4, 5, 6, 7, 8, 9 and 10). Also, exogenous GSH could not help to recover in hyphae form (Guedouari et al., 2014) in hyphae inducing media.

Thus, I had to consider the possibility of the relatively lowered MG levels by *GLR1* overexpression and its effects on morphogenesis and virulence in solid media. Based on our evidence, cellular MG and ROS content seemingly affect *Candida* morphogenesis by maintaining efficiently and minimally by *GLR1* overexpression (Figs. 4, 5 and 10; Baek, 2003). The results completely contrasted to GSH-depleted *Candida* and *Dictyostelium* mutants as described earlier (Kwak *et al.*, 2014; Kwak *et al.*, 2016; Kwak *et al.*, 2015). Therefore, to investigate both which signaling pathway is involved in the morphological changes and virulence attenuation and the involvement of cellular MG to the notified pathological alterations decisively caused by *GLR1* overexpression, the mRNA transcript levels of the conserved regulatory proteins in YB105 cells

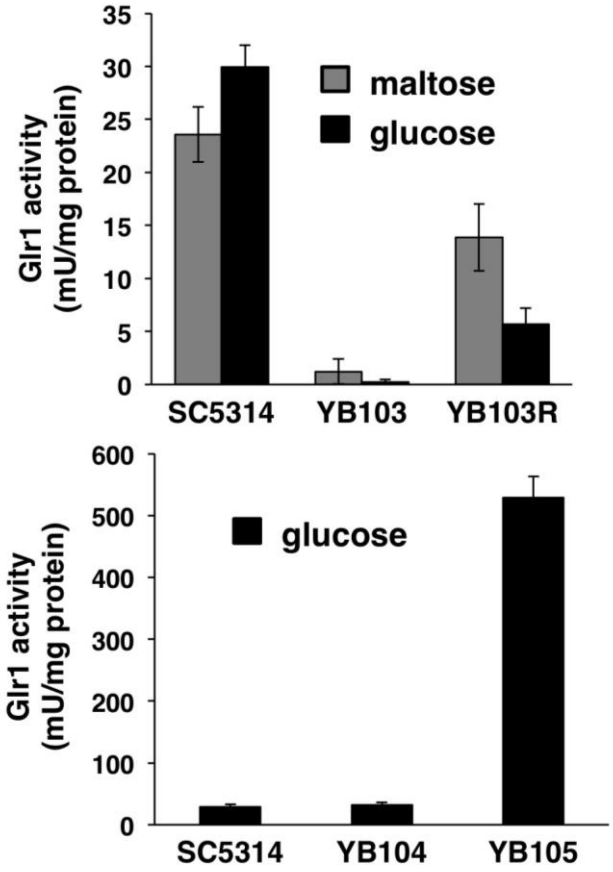


Fig. 7 The Glr1 activity of *GLR1*-overexpressing strain. The activity assay of Glr1 was examined spectrophotometrically using crude extracts as described in Materials and methods section. The decrease of the absorbance at 340 nm by the oxidation of NADPH was observed. Each strain of SC5314 (*GLR1/GLR1*), YB103 (*glr1/glr1::MAL2-GLR1::URA3*), YB103R (*GLR1::MAL2-GLR1::URA3*), YB104 (CAI4+pYPB1-ADHPt) and YB105 (CAI4+pGLR1-ADHPt) was grown exponentially in minimal media containing either glucose or maltose at 28°C. The values represent the averages ± standard deviation of three independent experiments.

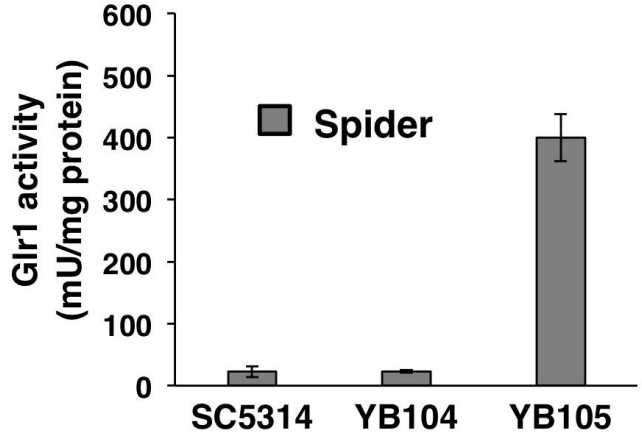


Fig. 8 The Glr1 activity of *GLR1*-overexpressing strain in Spider media. The activity assay of Glr1 was observed spectrophotometrically using crude extracts similar to as above experiments. Each strain of SC5314 (*GLR1/GLR1*), YB104 (CAI4+pYPB1-ADHPt) and YB105 (CAI4+pGLR1-ADHPt) was exponentially grown in Spider liquid broth at 37°C. The values represent the averages ± standard deviation of three independent experiments.

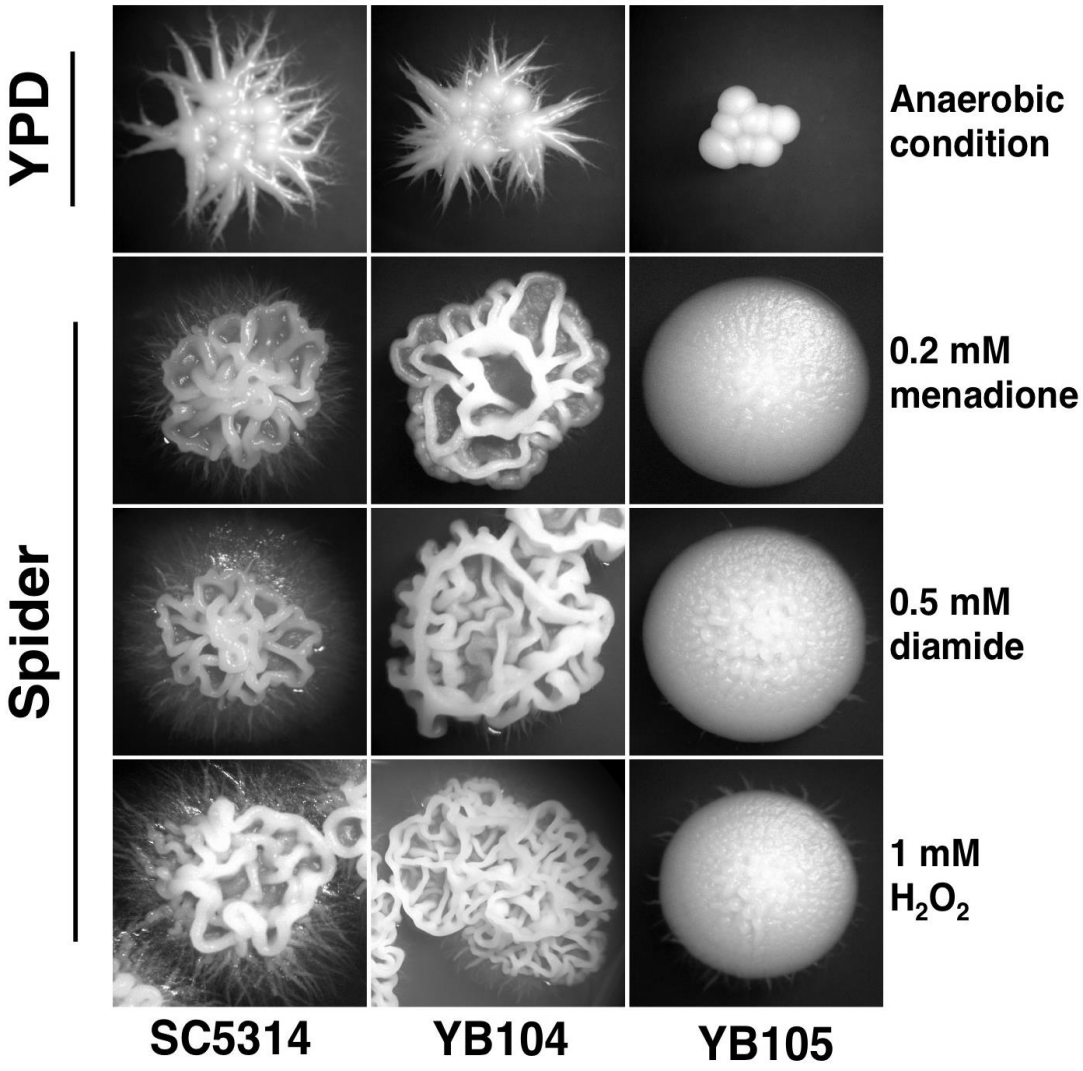


Fig. 9 Non-hyphal growth of *GLR1*-overexpressing strain against hypoxic and various oxidants. The *Candida* morphogenesis derived from anaerobic culture at room temperature. Each strain of SC5314 (*GLR1/GLR1*), YB104 (CAI4+pYPB1-ADHPt) and YB105 (CAI4+pGLR1-ADHPt) was maintained for 5 days at 28 or 37°C on YPD or Spider agar plates as described in Materials and Methods section.

were measured (Fig. 13). At the transcriptional levels, *EFG1* expression did not seem to change in either strain, but *CPH1* expression was significantly repressed in the YB105 strain (Fig. 13). In the case of the *TUP1* mRNA level, it showed a small reduction in expression. Based on the northern blot analysis, the *GLR1* seemed to induce morphological changes and to attenuate virulence under certain conditions, by modulating *CPH1* and *TUP1* expression as a complex. It was demonstrated that several kinds of transcriptional regulators might be involved in the morphological switch between the yeast and filamentous form associating with virulence change in *C. albicans* (Braun and Johnson, 1997; Braun and Johnson, 2000). Nevertheless, to reveal the exact protein expressions of these morphological regulators in *C. albicans*, western blot analysis were performed on Cph1, Efg1, and Tup1 expressions using *Candida* mutants (Hwang *et al.*, 2003), including JKC19 (*Δcph1*), HLC52 (*Δefg1*), HLC54 (*Δcph1/ Δefg1*), and BCa2-10 (*Δtup1*) (Figs. 13 and 14). Considering the defective in invasive filamentation particularly in the HLC52 and HLC54 strains (Fig. 14), the *GLR1* expression in these *EFG1*-deficient strains was remarkably increased (Fig. 15). It was indicated that the *GLR1*-mediated non-hyphal growth of *C. albicans* turned out to be strongly associated with both the repressed *CPH1* expression in the mRNA transcript level, however, concomitantly and predominantly with the repressed protein expression of Efg1.

Although these results showed the decreased transcriptional level of *CPH1* (Fig. 13) different from translational protein expression, non-significant Glr1 expression of JKC19 and BCa2-10 contrast to that of *EFG1* mutants, such as HLC52 and HLC54, was presumably supposed to be inhibited at the translational stage through an as yet non-identified mechanism. Additionally,

the genes (*ECE1*, *HYR1*, *ALS1*, and *INT1*) tested (Figs. 11A and 12) are known to be heavily dependent on *EFG1* (Braun and Johnson, 2000), which is exactly consistent with the results obtained here. The expression of *INT1* is contributed by *EFG1* and/or *CPH1* under different conditions (Sohn *et al.*, 2003). The expression patterns of regulatory genes in the YB105 strain (Fig. 13) were quite complicated, and it was hard to find a plausible explanation within the results above. However, these results might be explained the suggested model regulatory circuit by Braun and Johnson (2000) that a complex network of signaling pathway regulates filamentous growth through creating input of overlapping target genes. Clearly, *GLR1* overexpression was involved in regulation of hyphal growth and network of signal pathways of filamentous growth.

5. Effects of *GLR1* overexpression on cellular MG and ROS concentrations during hyphal formation

I previously postulated that the *GLR1* was required for cell growth and resistance to stresses. It was demonstrated by cellular GSH and along with MG content in budding or hyphal *GLR1* mutants (Figs 4, 5 and 10). Despite little contribution to decreasing hyphal capacity by GSH (Guedouari *et al.*, 2014), cellular GSH causes to alter GSH-dependent redox systems or other relevant metabolite pools (Kwak *et al.*, 2014; Kwak *et al.*, 2016; Kwak *et al.*, 2015; Michán and Pueyo, 2009; Swoboda *et al.*, 1994). For example, the elevated endogenous MG reciprocally increases cellular ROS with the declined GSH level in the *EAPX1*-deficient cells along with enhancement of cytochrome c peroxidase activity (Kwak *et al.*, 2015).

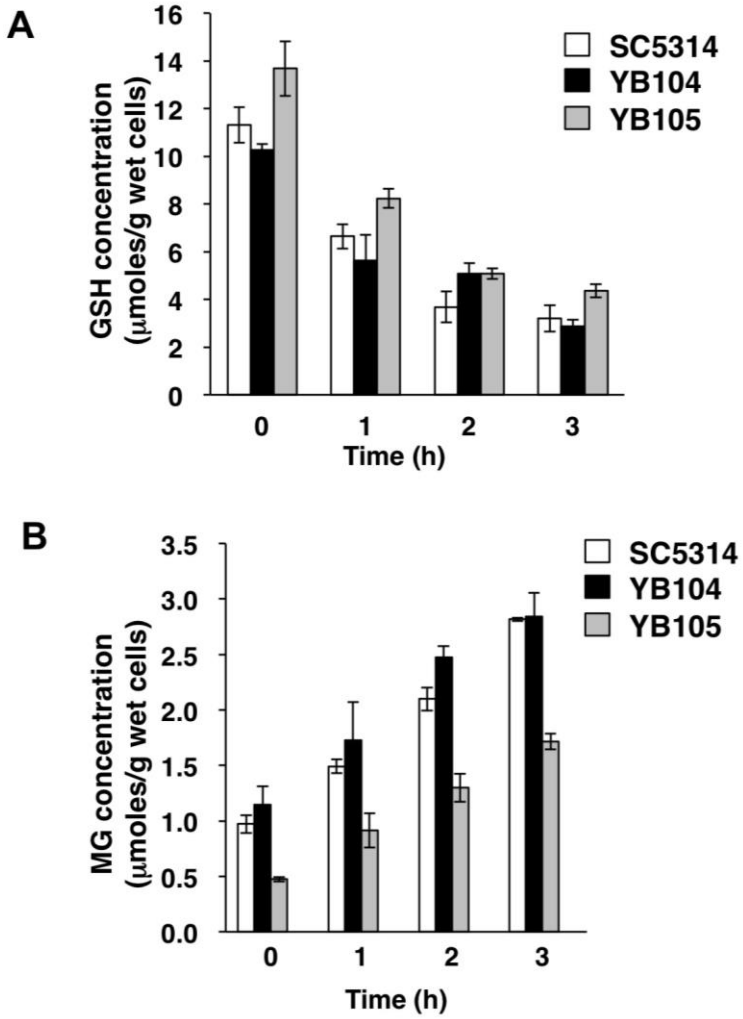


Fig. 10 The cellular GSH and MG concentrations of *GLR1*-overexpressing mutant. (A) The measurement of GSH and (B) MG in *Candida* cells, including SC5314 (*GLR1/GLR1*), YB104 (CAI4+pYPB1-ADHPt) and YB105 (CAI4+pGLR1-ADHPt), which were grown in Spider liquid medium at 37°C. The values represent the averages ± standard deviation of three independent experiments.

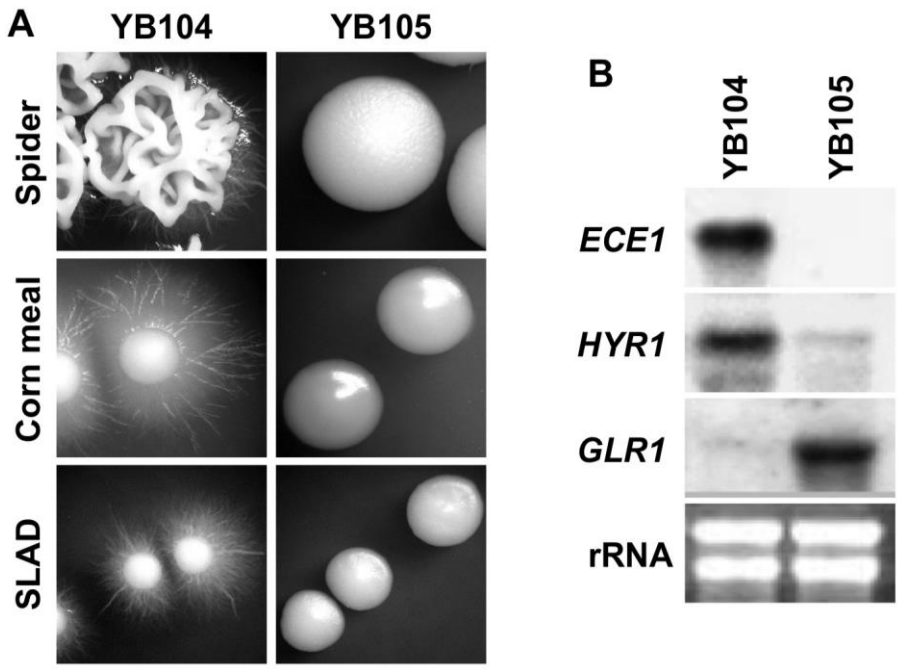


Fig. 11 Overexpression of *GLR1* results in defective filamentous growth of *C. albicans.* (A) Each strain of YB104 (CAI4+pYPB1-ADHPt) and YB105 (CAI4+pGLR1-ADHPt) was grown on solid Spider, Corn meal agar, and SLAD media at a density of 50-100 cells per plate for 5 days at 37°C (Spider, SLAD) or 28°C (corn meal agar). (B) Verification of hyphal growth by Northern blot analysis. Total RNAs were prepared from YB104 (lane 1) and YB105 (lane 2). Ten micrograms of RNA from each sample was subjected to Northern blot analysis using the indicated probes. The growth condition was described in Materials and methods section

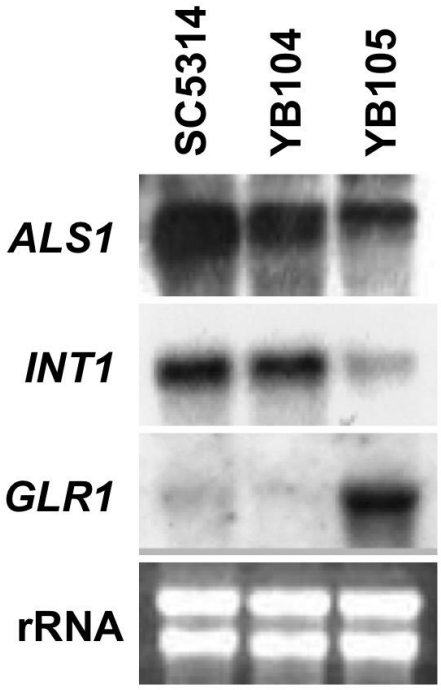


Fig. 12 mRNA expressions of virulence factors in *GLR1*-overexpressing strain. Total RNA from each strain, SC5314 (*GLR1/GLR1*) YB104 (CAI4+pYPB1-ADHPt), and YB105 (CAI4+pGLR1-ADHPt) under hypha-inducing condition was probed with the indicated genes. The hypha-specific inducing condition of cells was grown for 5 days at 37°C in spider agar plate as previously described.

Additionally, the *Dictyostelium odc*$^{-}$ cells, which remarkably increase in cellular MG, show severe GSH deficiency, particularly when depleting polyamines (Kwak *et al.*, 2015), and its endogenous/exogenous polyamines are proved to affect cellular MG along with GSH (Kwak *et al.*, 2016). These data entirely inferred that *Candida GLR1* overexpression could maximally reduce cellular MG and ROS production followed by exhibiting unique morphogenesis or altering virulence due to the high resistance to stresses with enhanced Glr1 activity (Scheme 4B and Fig. 8). These also corresponded that cellular GSH content might be possibly changed by other redox buffering mechanisms, including enzymatic- or non-enzymatic defense systems in fungi (Pócsi *et al.*, 2004). In *GLR1*-deficient YB103 cells, a parallel system such as Trx or Grx might be partially compensated for *GLR1* deficiency (da Silva Dantas *et al.*, 2010). These proteins have been elucidated to change cellular ROS generated by the host immune response along with the requirement for hyphal growth and virulence (Chaves *et al.*, 2007; Chaves and da Silva, 2012; da Silva Dantas *et al.*, 2010). However, the experimental evidence for the reciprocal changes in cellular MG and ROS that can alter the morphological transition derived from Glr1 activity (Kwak *et al.*, 2014; Kwak *et al.*, 2015), independently of the presence or absence of cellular GSH (Kwak *et al.*, 2016), has not been elucidated. Moreover, I previously hypothesized the minimal expressions of Trx- and Grx-related genes in budding YB105 cells because MG-scavenging functions associated with Trx or Grx system during dimorphic conversion have not been studied. Although MG could affect dysfunction of Trx mainly in endothelial or nerve cells in mammals (Dafre *et al.*, 2015; Oba *et al.*, 2012), these results were solely proven in MG-accumulating conditions similar to cells in the GSH-deficient *C. albicans* or *D. discoideum* cells (Choi *et al.*, 2008;

Kwak *et al.*, 2014; Kwak *et al.*, 2016). Moreover, because the YB105 cells were predicted to maintain against high oxidative stresses by the Glr1 activity with the high ratio of GSH/GSSG, the Glr1 activity might sustain at the significant lower basal level of cellular MG content. Upon these preliminary findings, the investigation of the reciprocal effect between MG, ROS, and GSH reflecting *Candida* dimorphism especially by Glr1 activity was studied.

Therefore, due to the repressed Efg1-mediated non-hyphal phenotype triggered by the *GLR1* expression, cellular MG as a candidate for another morphogenic factor was preferentially observed along with GSH and ROS in YB105 strain. Contrast to significantly decreased and increased cellular GSH and MG undergoing filamentation in liquid medium (Fig. 10), cellular GSH content was remarkably higher than reference strains concomitantly with minimally retained MG and ROS content in YB105 strain in both non-hyphal and hyphal-inducing conditions (Figs. 16 and 17). However, we could not measure MG levels in solid SLAD and Corn meal due to the limitation of collecting appropriate amounts of samples (Fig. 17A). As expected, we observed that intracellular GSH increased significantly compared to the reference strain YB104 (Fig. 16), and cellular MG and ROS production were remarkably decreased in a manner that was inversely proportional to GSH level in YB105 cells during filamentous growth (Fig. 17). Based on these results, YB105 by *GLR1* overexpression acted as an antioxidant strain by scavenging cellular MG and ROS levels corresponding to the previous investigations. Furthermore, cellular ROS content increased and dependently expressed during MG biosynthesis in fungi (Kwak *et al.*, 2014; Kwak *et al.*, 2015), thereby promoted GSH depletion in mammals (Seo *et al.*, 2014) although the parallel mechanism was maintained between MG, ROS, H_2O_2, and ASC or EASC in

yeast (Huh *et al.*, 2001; Huh *et al.*, 2008). In plants, it was also demonstrated that ROS or MG production accompanied GSH depletion (Hossain *et al.*, 2012). Thus, the intracellular *GLR1* function might be parallel to the Trx and Grx. Both these proteins were observed to be involved particularly in regulating the oxidative stress in *Candida* physiology because *C. albicans* gene sets related to various induced stresses did not always respond to corresponding stresses using the Trx and Grx systems (Enjalbert *et al.*, 2003).

These findings suggested that the GSH-deficient/-overproducing cells can consume cellular MG by activation of the γ-glutamyl cycle (Choi *et al.*, 2008), which has been elucidated as an initial MG degradation pathway of glyoxalases in prokaryotes and eukaryotes (Inoue *et al.*, 2011; Murata *et al.*, 1989). Thus, I concluded that the decreased cellular MG or ROS levels in the YB105 strain during hyphal growth regulated by the reduced GSH by *GLR1* overexpression and contribution of low GSH content in cells to filamentation.

6. *GLR1* alters gene expressions of glycolytic enzymes concomitantly with *ADH1* during hyphal growth

Our laboratory members previously demonstrated that the GSH-depleted *ADH1*-deficient cells were bearing defects in dimorphic switching (Kwak *et al.*, 2014), corresponding that the gene expressions of *ADH1* or glycolytic enzymes might contribute to the hyphal growth or virulence in *C. albicans* (Swoboda *et al.*, 1994). However, it was previously noted that the glycolytic mRNA levels during morphogenesis did not correlate with cellular dimorphism under certain experimental conditions, including changes in the culture temperature and hyphae-inducing medium (Swoboda *et al.*, 1994). Nevertheless, *ADH1* expression seemingly contributed to scavenge cellular MG

particularly in the GSH- and *ADH1*-deficient double disruptants, which corresponded to cells regarding the maximal condition for the endogenous MG accumulation (Kwak *et al.*, 2014). Moreover, it was also elucidated that cellular ROS increases especially in high-glucose medium, which could accelerate MG biosynthesis in cells and cause to poor cell growth due to the GSH deficiency in *Dictyostelium* (Kwak *et al.*, 2016). As previous reports, cellular MG biosynthesis during glycolytic pathways by the autoxidation of glyceraldehyde and monosaccharides (Thornalley *et al.*, 1984) could be involved in the altered production of a series of glycolytic enzymes, including fructose 1,6-bisphosphate aldolase (FBA1), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and phosphoglycerate kinase (Leoncini *et al.*, 1980). These previous investigations inferred that GSH-synthesizing- and glycolytic enzymes might be strongly associated with GSH biosynthesis followed by MG scavenging functions in cells that might undergo morphological changes.

Therefore, I had to consider the possibility that GSH and MG biosynthesis reciprocally regulated by Glr1 during filamentous growth. Also, it might affect expressions of a set of genes encoding glycolytic enzymes and *ADH1*, which can catalyze the conversion of MG to pyruvate (Kwak *et al.*, 2014) associated with *GLR1* as shown in the *ADH1*-deficient cells. To verify the interplay between glycolytic enzymes, ADH1, and GLR1, *Candida FBA1* and *TDH1* overexpressed mutants were used (Table 1), respectively. Unexpectedly, the mRNA profile of *ADH1* was remarkably increased in YB105 cells, and the transcript level of *FBA1* and *TDH3* also displayed the significant increase compared to the reference experiment (Fig. 18, 19 and 20). This result fully indicated that *GLR1* expression coincidentally could regulate *FBA1* and *TDH3*

gene expressions, which could alter the glycolytic metabolites, followed by changes in the cellular GSH and MG content.

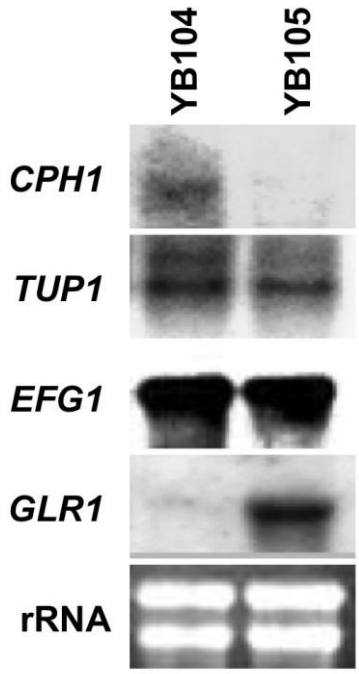


Fig. 13 Changes in regulatory gene expression caused by *GLR1* overexpression. Total RNA from each strain of YB104 (CAI4+pYPB1-ADHPt) and YB105 (CAI4+pGLR1-ADHPt) under the hyphae-inducing condition was probed with the indicated genes. The hyphae-specific inducing condition was maintained for 5 days at 37°C on Spider agar plates as described in Materials and methods section.

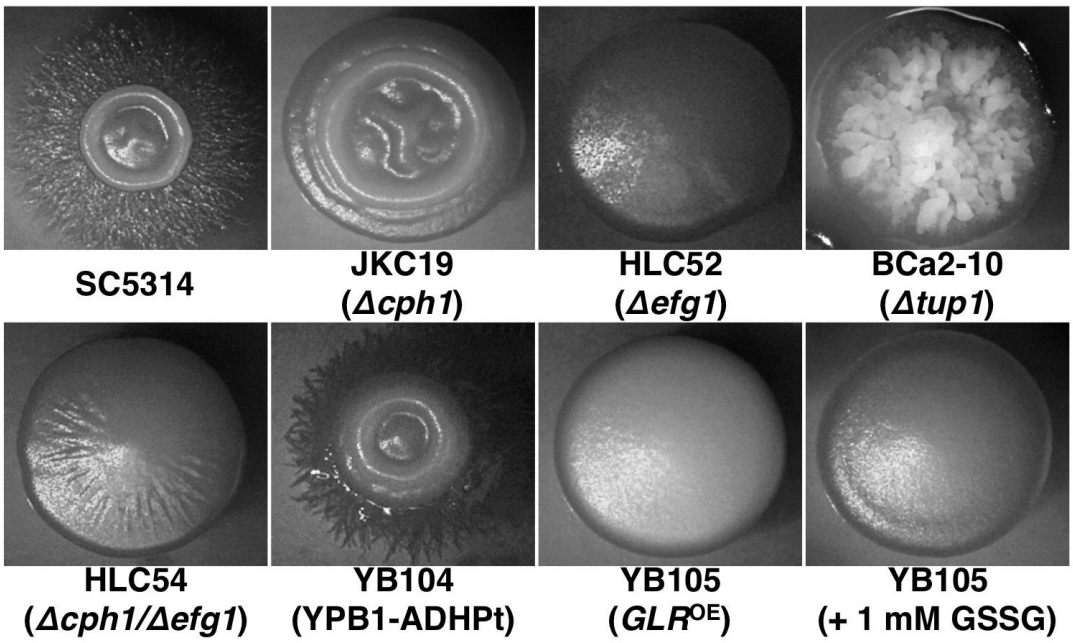


Fig. 14 Colony morphology of various mutants involved in *CPH1*- and *EFG1*-mediated signaling pathways. The Ura^+ cells, including SC5314 (*GLR1/GLR1*), JKC19 (*Δcph1*), HLC52 (*Δefg1*), HLC54 (*Δcph1/ Δefg1*), BCa2-10 (*Δtup1*), YB104 (CAI4+YPB1-ADHPt) and YB105 (CAI4+ pGLR1-ADHPt), were incubated on solid Spider medium at 37°C for 5 days.

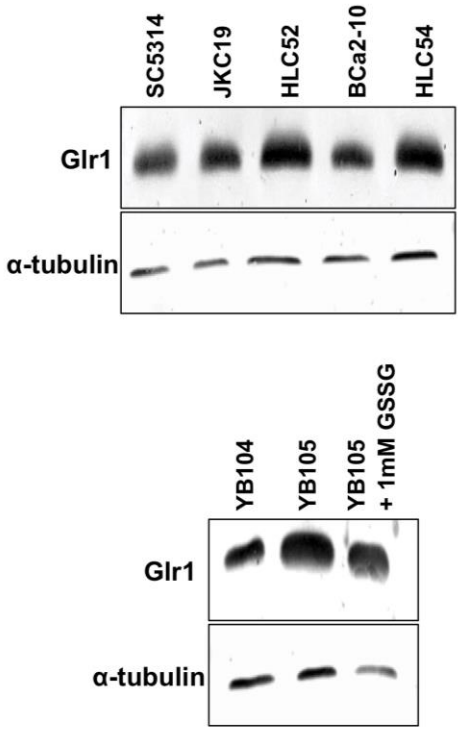


Fig. 15 The expression of GLR1 remarkably repressed *CPH1*- and *EFG1*-mediated signaling pathways affecting yeast morphology. SC5314 (*GLR1/GLR1*), JKC19 (*Δcph1*), HLC52 (*Δefg1*), HLC54 (*Δcph1/ Δefg1*), BCa2-10 (*Δtup1*), YB104 (CAI4+YPB1-ADHPt) and YB105 (CAI4+ pGLR1-ADHPt) were incubated on solid Spider medium at 37°C for 5 days. The scraped cells from colonies were used to observe the protein expression levels of GLR1 using western blot analysis. 100 μg of crude extracts from each strain was subjected to western blot analysis as described in Material and Methods.

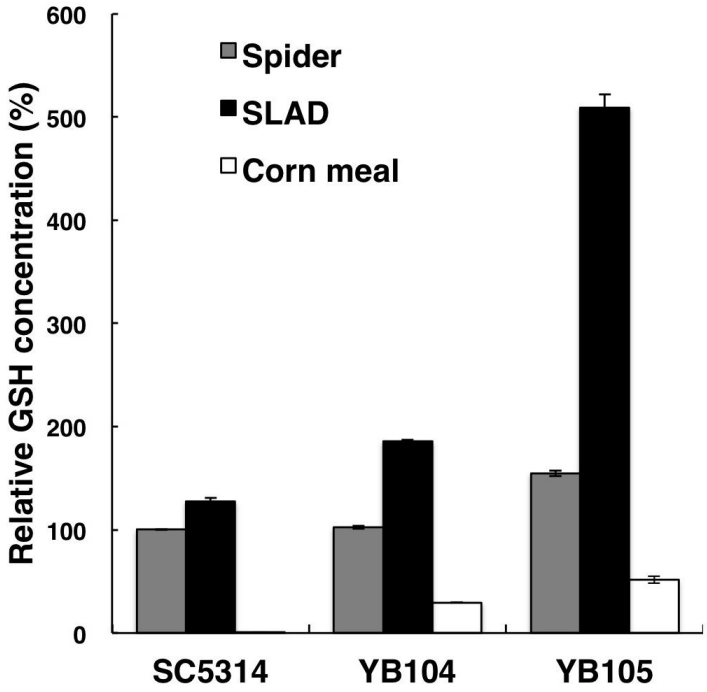


Fig. 16 The cellular concentration of GSH in *GLR1*-overexpressing strain during hyphal formation in *C. albicans*. Each strain of SC5314 (*GLR1*/*GLR1*), YB104 (CAI4+pYPB1-ADHPt) and YB105 (CAI4+pGLR1-ADHPt) was grown on solid Spider, Corn meal agar, and SLAD media at a density of 50-100 cells per plate for 5 days at 37°C (Spider, SLAD) or 28°C (corn meal agar). The scraped cells from colonies were used to measure intracellular GSH using analytic HPLC. The values represent the averages ± standard deviation of three independent experiments.

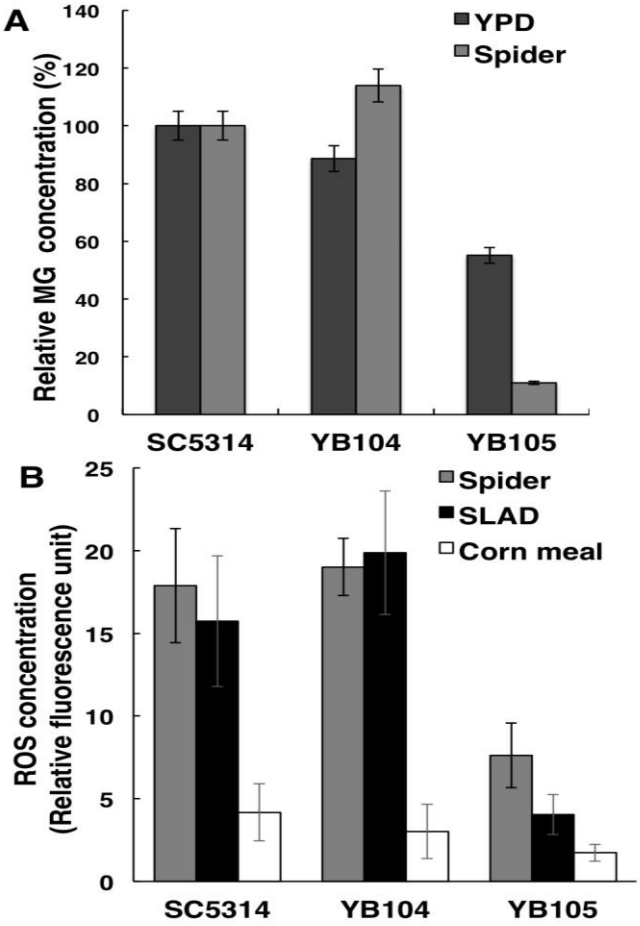


Fig. 17 The cellular concentration of MG and ROS in *GLR1*-overexpressing strain during hyphal formation in *C. albicans*. Each strain of SC5314 (*GLR1*/*GLR1*), YB104 (CAI4+pYPB1-ADHPt) and YB105 (CAI4+pGLR1-ADHPt) was grown on solid Spider, Corn meal agar, and SLAD media at a density of 50-100 cells per plate for 5 days at 37°C (Spider, SLAD) or 28°C (corn meal agar). The scraped cells from colonies were used to measure (A) intracellular MG using analytic HPLC and (B) relative ROS concentrations using a fluorescence spectrometry. The values represent the averages ± standard deviation of three independent experiments.

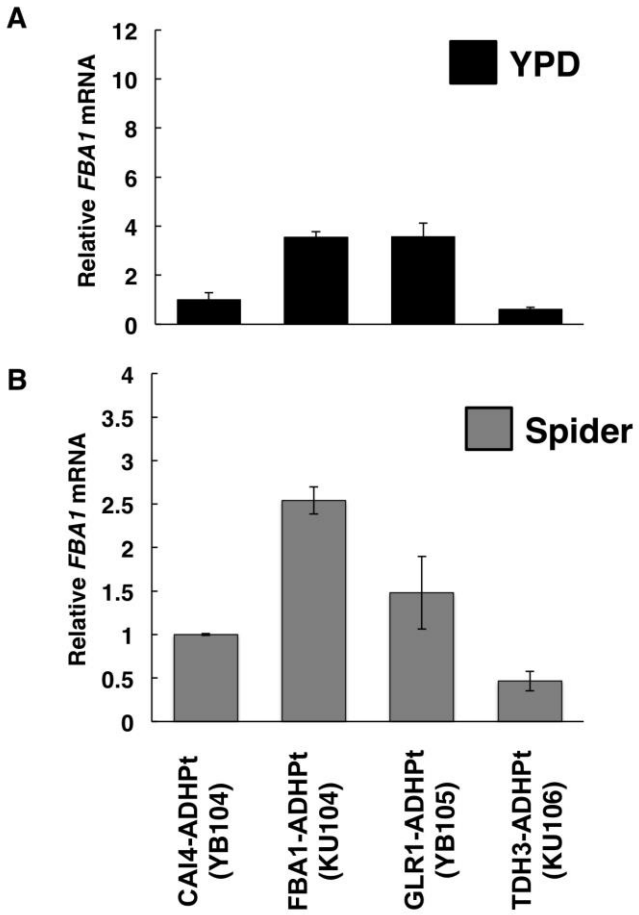


Fig. 18 The effect of *GLR1*-overexpressing cells on *FBA1* gene expressions during budding and hyphal growth by using RT-PCR. The relative amount of mRNA profiles of glycolytic enzymes *FBA1*, as indicated in (A) YPD and (B) Spider solid medium, respectively. The values represent the averages ± standard deviation of three independent experiments. All experimental procedures are described in the Materials and methods section.

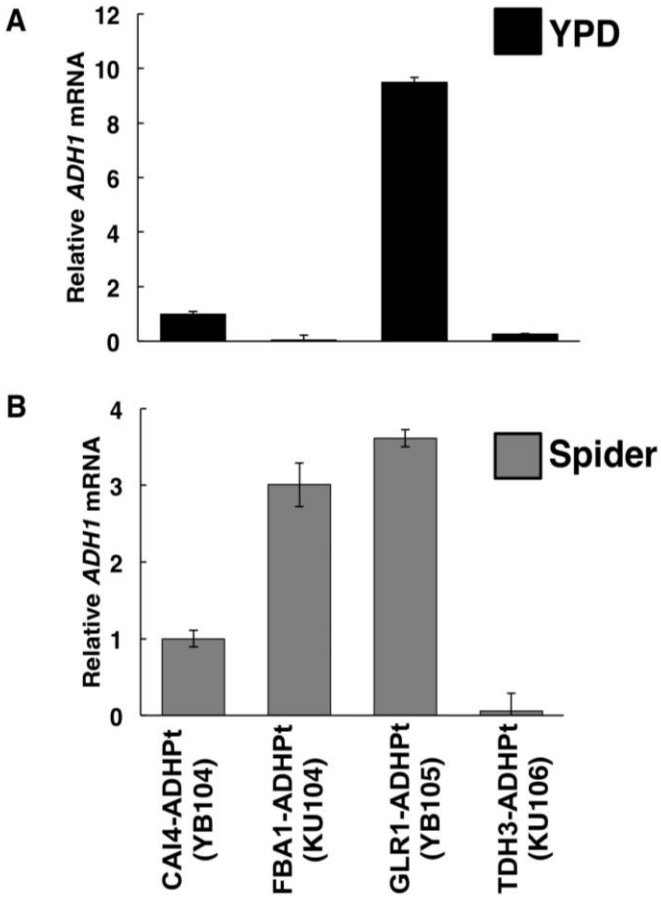


Fig. 19 The effect of *GLR1*-overexpressing cells on *ADH1* gene expressions during budding and hyphal growth by using RT-PCR. The relative amount of mRNA profiles of *ADH1*, as indicated in (A) YPD and (B) Spider solid medium, respectively. The values represent the averages ± standard deviation of three independent experiments. All experimental procedures are described in the Materials and methods section.

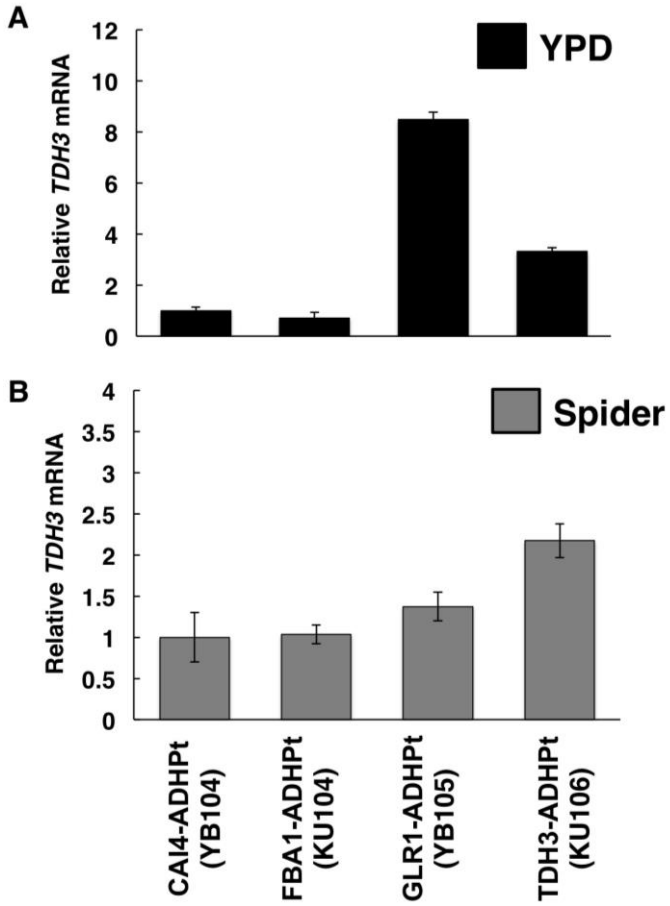


Fig. 20 The effect of *GLR1*-overexpressing cells on *TDH3* gene expressions during budding and hyphal growth by using RT-PCR. The relative amount of mRNA profiles of *TDH3*, as indicated in (A) YPD and (B) Spider solid medium, respectively. The values represent the averages ± standard deviation of three independent experiments. All experimental procedures are described in the Materials and methods section.

IV. Conclusion

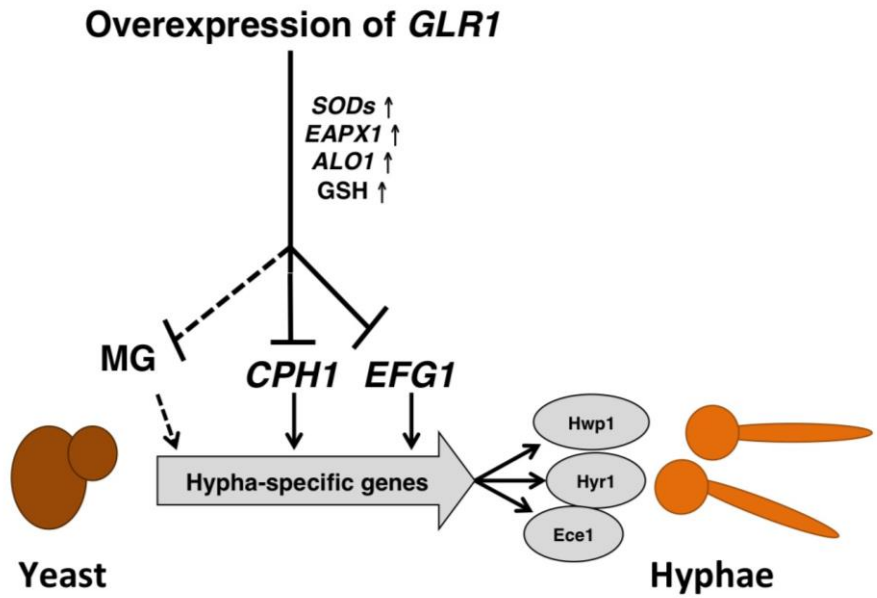


Fig. 21 Proposed model of the effect of *GLR1* in the differentiation of *C. albicans*. Along with other antioxidant gene expressions, the expression of *GLR1* represses Cph1-/Efg1-dependent signaling pathway to maintain budding yeast form by decreasing cellular MG, which suggested as a possible cue to initiate the morphological transition. How *GLR1* could decrease MG, mainly derived from glycolysis, when *Candida* cell underwent the morphological transition from yeasts to hyphae remains elusive.

GSH is an effective enzymatic or non-enzymatic antioxidant, and *Candida* Glr1 activity is required for maintaining antioxidant activity for viability. When *GLR1* gene was overexpressed in *C. albicans*, this organism showed enhanced resistance to various stresses. *GLR1* gene is indispensable for the proper growth of budding *C. albicans* and renders cells resistant to oxidative stresses dissimilar to other yeasts. Another remarkable result was that overexpression of *GLR1* results in completely impaired hyphal growth and virulence in *C. albicans*. The morphogenetic switching in *C. albicans* has been extensively studied because of its close relationship to virulence. For this reason, we propose a complex network that is involved in hyphal growth and virulence by *GLR1* (Fig. 21). The model represents that both non-hyphal forming *GLR1*-overexpressing YB105 and GSH-supplemented *EAPX1*-deficient/overexpressing or GSH-depleted *ADH1*-deficient cells might affect by the cellular MG and ROS. Additionally, further characterization had to be considered to identify whether Glr1 exerts its effect on morphogenesis through an already known signaling pathway involving either *CPH1* or *EFG1* or through an unknown pathway. Interestingly, *GLR1* overexpression in *C. albicans* caused non-filamentous growth, close to budding yeast, in several hypha-inducing conditions. However, cellular GSH did not contribute to hyphal growth, suggesting that *GLR1* expression is changed in response to environmental stresses, leading to regulate changes in the expression of genes related to morphogenesis, especially by Efg1 repression (Figs. 9, 10, 11, 12, 13, 14, and 15). Surprisingly, *Candida* dimorphism and virulence were co-regulated by *GLR1* as a negative regulator during cell differentiation. It is proved through other reciprocal gene expressions of *ADH1*, *FBA1*, and *TDH3* under the control of Efg1 and/or Cph1 signaling pathway along with the

cellular changes of MG and GSH (Figs. 16, 17, 18, 19 and 20). There is a possibility that *GLR1*-mediated Efg1 down-regulatory mechanism is involved and/or a complex network of signal pathways may function together. Also, further studies are needed to elucidate the origin of MG since it seems to affect multiple pathways. Demonstrating how *GLR1* expression influences multiple signaling pathways and the source of MG can help verify attractive targets for antifungal drugs.

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국 문 초 록

글루타티온 환원효소는 산화형 글루타티온을 환원시킴으로써 세포 내의 환원된 글루타티온 양을 유지하는데 매우 효율적이다. 글루타티티온은 세포 생장과 분열에 요구되며 이것의 생합성 효소 결핍은 해당 과정으로 부터 생성된 메틸글리옥살의 축적을 야기한다는 것은 이미 밝혀진바 있다. 그러나 효모의 형태학적 측면에서 Cph1/Efg1 을 매개로 한 신호 전달 경로의 조절과 글루타티온 환원효소에 의한 해당 과정으로부터의 메틸글리옥살 생성 간의 상호관계를 비롯하여 메틸글리옥살의 생성 기원에 대한 실험적 증거는 불분명하다.

GLR1 의 유전자를 CaMAL2 프로모터를 사용하여 조건적으로 저해시켰을시 외생적 싸이올을 추가했음에도 불구하고 생존이 불가능하였다. *GRL1* 과발현 세포는 사상체 형성동안 비생장 균사를 띄었고 산화제에 저항성을 보였으며 세포 내 메틸글리옥살의 양이 현저히 감소하였다. 또한, Efg1 신호 전달 흐름의 현저한 억제와 함께 FBA1, TDH3, 그리고 알코올 탈수소효소 (ADH1)을 포함한 에너지 생성 효소를 암호화하고 있는 유전자 발현의 증가를 수반하였다.

본 연구에서 *GLR1* 에 의해 유발된 *Efg1* 매개 신호 변환의 억제는 해당과정 효소와 *ADH1* 의 유전자 발현이 증가함에 따라 메틸글리옥살의 기본 수준을 유지함으로써 형태 변환과 독성을 엄격하게 줄인다는 것이 확인되었다. 이러한 결과는 *GLR1* 발현에 의한 Efg1 신호 전달 흐름의 하향조절성 기작과 해당 과정에서 생성되는 메틸글리옥살의 양을 조절

방법은 *C. albicans* 의 독성을 타겟으로 하는 항진균제 개발에 그 역할을 할 가능성이 사료된다.

주요어; 글루타티온 환원효소; 글루타티온; 메틸글리옥살; 과당-1,6-인산 알돌라아제; Efg1-mediated cyclic AMP/protein kinase A 경로; 캔디다 알비칸