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ORIGINAL PAPER

Glutathione reductase from *Brassica rapa* affects tolerance and the redox state but not fermentation ability in response to oxidative stress in genetically modified *Saccharomyces cerevisiae*

Ho-Sung Yoon · Sun-Young Shin · Young-Saeng Kim · Il-Sup Kim

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Abstract To determine whether the exogenous expression of glutathione reductase (GR) from *Brassica rapa* subsp. *pekinensis* (BrGR) can reduce the deleterious effects of unfavorable conditions, we constructed a transgenic *Saccharomyces cerevisiae* strain bearing the GR gene cloned into the yeast expression vector, pVTU260. BrGR expression was confirmed by semi reverse transcriptase-polymerase chain reaction (RT-PCR) analysis, immunoblotting analysis and an enzyme assay. Ectopic BrGR-expression improved cellular glutathione (GSH) homeostasis after higher GSH accumulation in the transgenic yeast than in the wild-type yeast under H_2O_2-induced oxidative stress. The BrGR-expressing yeast strain induced the activation of metabolic enzymes (Hxt, G6PDH, GAPDH and Ald), antioxidant systems (Gpx, Trx2, Trx3, Trr1, Tsa1 and porin) and molecular chaperones (Hsp104, Hsp90, Hsp70, Hsp42, Hsp26, Grp, Sti1 and Zpr1), which led to lower oxidative protein damage after a reduction in the level of cellular ROS in the BrGR-expressing yeast strain exposed to H_2O_2 than in the wild-type yeast strain. BrGR-expression increased the ability to adapt and recover from H_2O_2-induced oxidative stress and various stressors, including heat shock, menadione, *tert*-butyl hydroperoxide, heavy metals, sodium dodecyl sulfate, ethanol and NaCl, but did not affect fermentation capacity. These results suggest that ectopic BrGR expression confers acquired tolerance by improving proteostasis and redox homeostasis through co-activation of various cell rescue proteins against ROS-induced oxidative stress in yeast cells.

Keywords BrGR expression · Oxidative stress · Stress tolerance · Redox state · Cell rescue proteins

Introduction

Oxidative stress is generated from the toxic levels of oxygen-derived reactive oxygen species (ROSs) such as singlet oxygen, superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and the highly reactive hydroxyl radical ($OH^\cdot$) via the metal-catalyzed Haber–Weiss and Fenton reaction (Jamieson 1998; Costa and Moradas-Ferreira 2001). These ROSs are the result from not only normal metabolic processes, including the energy generation process of aerobic respiration and β-oxidation of fatty acids, but also exposure to abiotic stresses conferring heat, osmotic pressure and irradiation, or chemical stress including ethanol, H_2O_2, menadione (MD), heavy metals, xenobiotics, etc. (Pereira et al. 2001). The produced ROSs can lead to a wide range of damage of cellular biological molecules, such as DNA fragmentation and mutations, lipid peroxidation, the disassembly of iron-sulfur clusters, disulfide bond formation, and other types of protein oxidation (Carmel-Harel and Storz 2000).

To overcome unfavorable environments, aerobic organisms have developed a wide range of antioxidant systems. Tripeptide glutathione (GSH, gamma-L-glutamyl-L-cysteinylglycine), is the most abundant low-molecular-weight thiol in most organisms participating in multiple metabolic processes from bacteria to animals. GSH plays a variety of physiological roles in stress: intracellular redox state regulation, inactivation/detoxification of reactive oxygen species

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H.-S. Yoon · S.-Y. Shin · Y.-S. Kim · I.-S. Kim (✉)
Department of Biology, Kyungpook National University, #1370 Sankyuk-dong, Buk-gu, Daegu 702-701, Republic of Korea
e-mail: 92kis@hanmail.net

(ROS), export into the vacuole by formation of GSH-conjugates for the detoxification of xenobiotics and heavy metals such as cadmium, participation in the synthesis of DNA precursors (via ribonucleotide reductase), protein folding and amino acid transport, (Wheeler and Grant 2004) and the storage of sulfur and cysteine affording up to 90% of the non-proteinic sulfur in the cell (Mendoza-Cozatl et al. 2005). One of the major functions of GSH is in bio-reductive reactions as an important line of defense against ROS. The redox active sulfhydryl group of GSH can protect cells from ROS by directly scavenging free radicals and acting as a cofactor for antioxidant enzymes such as glutathione peroxidases (Wheeler and Grant 2004).

Glutathione reductase (GR, EC 1.6.4.2) is a homodimeric flavoprotein which catalyzes the reduction of oxidized glutathione (GSSG) to the reduced form (GSH) in the presence of NADPH as a reducing power. GR has a relation with diverse cellular phenomena, including the defensive response against free radicals and ROS as well as protein and DNA biosynthesis, maintaining a high ratio of GSH/GSSG (Kim et al. 2010). GSSG is usually generated through the oxidation of GSH by oxidants, such as ROS, produced by oxidative stress (Jamieson 1998). Accordingly, the GSH/GSSG ratio is decreased by oxidative stress, suggesting that the GSH and GSSG constitute a cellular redox buffer. Mutants with a *GLR1* knockout in yeast and GR knockout in mice are viable, have a high accumulation of GSSG, and are hypersensitive to oxidants such as menadione, resulting in a state of thiol oxidative stress, and implying that GR contributes to the defense against ROS-induced oxidative damage (Tan et al. 2010). Up-regulation and down-regulation of GR was identified to cause various changes in many types of cells. Transgenic plants (Foyer et al. 1995) and *Drosophila* (Mockett et al. 1999) that overexpress GR, were shown to be more resistant to environmental stress than the control wild types.

Brassica vegetables, which are commonly known as crucifers, include a variety of agricultural crops, which play a significant role in worldwide vegetable production and consumption. Brassica vegetables are low-fat and low-protein foods and have a high content in fiber and minerals. Besides, they show high quantities of antioxidant chemicals, such as glutathione, vitamins and phenolic compounds, which are widely studied for their beneficial properties (Francisco et al. 2011). The cDNA of the GR gene from *Brassia rapa* (BrGR) has been cloned (Lee et al. 1998; Lee et al. 2002). BrGR over-expression was found to increase upon exposure to oxidative stress such as ozone and paraquat (Yoon et al. 2005), and H_2O_2-induced oxidative stress (Kim et al. 2009) in *Escherichia coli* using the *E. coli* BL21 system for basic biochemical studies. However, there is little molecular and structural information available for the GR protein of *B. rapa*, even though it would be necessary to obtain valid information on how BrGR is regulated in responses to environmental challenges. In this study, we have shown that ectopic overexpression of glutathione reductase (GR) from *Brassica rapa* subsp. *pekinensis* (BrGR) results in acquired tolerance and improved redox state of *Ssccharomyces cerevisiae* in response to ROS-induced oxidative stress by maintaining a high GSH/GSSG ratio and co-activating various cell rescue proteins. BrGR expression does not increase fermentation ability on YG medium.

Materials and methods

Construction of the BrGR-expressing recombinant yeast strain

Total RNA was isolated from *Brassia rapa* leaves. The cDNA probe was prepared by reverse transcriptase-polymerase chain reaction (RT-PCR). The BrGR coding region was amplified from cDNA by PCR using *Taq* and *Pfu* polymerase (Roche, Germany). PCR reaction conditions were as follows: initial denaturation cycle was 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 2 min, and a final extension step of 7 min at 72°C. The primers utilized for the PCR cloning of BrGR gene were 5′-GATTCTGCACAA**CCATGG**CGAGGAA GATGC-3′ (*Nco*I site underlined) and 5′-GAGTGTGA-ATT**GGATCC**AAAGATAATGC-3′ (*BamH*I site underlined) as sense and antisense primers, respectively. The PCR product was inserted into the TOPO TA cloning vector (Invitrogen, USA). The cloned plasmid was sequenced using the M13 primer set to confirm that no PCR-induced mutations were introduced (Genotech Inc., Korea). The cloned plasmid DNA was ligated into the yeast expression vector pVTU260 (EUROSCARF, Germany) digested with the appropriate restriction enzymes. The constructed plasmid was named pVTU260::BrGR. The *E. coli* DH5α strain that had been transformed with the pVTU260::BrGR plasmid was used to amplify the plasmid DNA. The plasmid DNA was isolated by a plasmid isolation kit (Nucleogen Inc., Korea). The cells of *S. cerevisiae* BY4741 were grown overnight in YPD medium, and were cultured for 4 h at 30°C until cultures reached approximately an OD_{600} of 1.5, and were then transformed with pVTU260::BrGR via the PEG/LiCl method (Gietz and Woods 2001). Transformants were selected as growing cells on yeast synthetic drop-out agar medium containing 2% glucose but lacking uracil, at 30°C for 2–3 days. Colonies were then restreaked and incubated under the same conditions. Positively transformed colonies with the pVTU260::BrGR were able to grow medium lacking uracil. Strains used in this study were listed in Table 1.

Semi-quantitative RT-PCR analysis

The total RNA from mid-log phase yeast cells (OD_{600} = 2.0) was obtained using the *hot phenol* method as previously described (Schmitt et al. 1990). First-strand cDNA was synthesized by incubating 1 μg of total RNA with RT-PreMix (Bioneer Inc., Korea) and oligo$(dT)_{18}$ at 42°C for 1 h in a 20 μl reaction volume. Subsequently, the BrGR gene was amplified from epimastigote cDNA using a gene-specific primer. The oligonucleotides used were BrGR-F and BrGR-R; the sequences were 5′-TGGGAAATCAATG GGAATGT-3′ and 5′-CCGTCCAGAAATGGTGTTCT-3′, respectively. The PCR amplicon was separated onto a 1.2% agarose gel in 0.5X TBE buffer, stained with ethidium bromide, visualized and then, photographed. The PCR amplicon *PDA1* (Wenzel et al. 1995) was used as a housekeeping control.

Stress tolerance assay

To measure the growth rate under various stresses, cells (1×10^5 cells per ml) grown overnight were inoculated in YPD medium containing each of the following: 3.5 mM H_2O_2 and 40 μM MD, 1.0 mM *tert*-butyl hydroperoxide (t-BOOH), 50 μM cadmium chloride ($CdCl_2$), 0.2% SDS, 10.0 mM zinc chloride ($ZnCl_2$), 5.0 mM copper sulfate ($CuSO_4$), and 1.0 M sodium chloride (NaCl), each in its own YPD medium. Optical density was measured at 600 nm at 2 h intervals for the indicated time. For the spotting assay, once reaching the mid-log phase (A_{600} = 3.5), cell aliquots were exposed to 30.0 mM H_2O_2, 0.2 mM MD, 10.0 mM t-BOOH, 5.0 mM $CdCl_2$, 1.0% SDS, 0.1 M $ZnCl_2$, 20.0 mM $CuSO_4$, 0-20% ethanol, and 5.0 M NaCl, respectively, for 1 h at 30°C (having been properly shaken), and serially diluted tenfold with distilled water. 5.0 μl of diluted solutions were loaded onto YPD agar medium. To induce heat shock, the cells were treated for 20 min at 55°C. Stress recovery was performed as described above. In the case of glucose shock, 5.0 μl each of diluted solutions was loaded onto yeast extract-peptone-dextrose (YPD) agar medium containing 2% glucose or YG medium containing 5, 10, 15, or 20% glucose. Each experiment was performed 3 times and representative results are shown.

Protein preparation

Crude protein extracts were prepared using the glass bead method. Cells were exposed to heat shock (55°C for 10 min), 30.0 mM H_2O_2, 0.2 mM MD, 10.0 mM t-BOOH, 5.0 mM $CdCl_2$, 1.0% SDS, 0.1 M $ZnCl_2$, 20.0 mM $CuSO_4$, and 5.0 M NaCl, respectively, for 1 h at 30°C (having been properly shaken), washed with a cold-PBS buffer containing 0.85% NaCl three times, and resuspended in a lysis buffer containing 50 mM HEPES, pH 7.5, 5% glycerol, 1 mM PMSF, 2 μM pepstatin A and a protease inhibitor cocktail, with an equal volume of glass beads (425–600 μm; Sigma, USA). After vigorously vortexing each 5 times for 1 min at 2-min intervals on ice, the cleared protein extracts were collected by centrifugation at 13,000 rpm for 20 min at 4°C. The protein concentration was determined by Bradford assay using Protein Dye Reagent (Bio-Rad, USA).

Western blot analysis

The protein extract (20 μg) was loaded onto a 12% or 15% SDS-PAGE gel, and run at 70 V (Laemmli 1970). The gel was electrophoretically transferred to a PVDF membrane after SDS-PAGE. The membranes were blocked for 1 h at room temperature, and incubated overnight at 4°C with the following primary antibodies: anti-glutathione reductase and glutathione peroxidase (Agrisera, Sweden), His-tag (Millipore, USA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Ab frontier, Korea), glucose-6-phosphate dehydrogenase (G6PDH) and Zpr1 (Sigma, USA), porin (Invitrogen, USA), tubulin and malondialdehyde (MDA) (SantaCruz, USA), and Hsp104 and Hsp60 (Stressgen, Canada). Anti-Hsp90, Hsp42, Hsp26, Grp, Sti, aldehyde dehydrogenase (Ald), hexokinase (Hxk), thioredox (Trx2 and Trx3), thioredoxin reductase (Trr1), thioredoxin peroxidase (Tsa1) antibodies were used as reported previously (Kim et al. 2011). Anti-tubulin antibody was used as a housekeeping control. Blots were washed, and incubated with horseradish peroxidase coupled to anti-rabbit, goat or mouse (SantaCruz, USA) secondary antibody for 1.5 h at room temperature. After washing, the signal was visualized using ECL Western blotting detection reagent (GE Healthcare, USA).

Glutathione reductase activity

Cells were grown to the mid-log or stationary phases in YPD and YG media and harvested by centrifugation. GR activity was assayed spectrophotometrically. The assay was done at room temperature with a reaction mixture (total 1 ml) containing 50 mM phosphate buffer (pH 7.8), 0.5 mM GSSG, 0.1 mM NADPH, and crude extract from the yeast cells. The absorbance was measured at 340 nm. The activity was calculated using the absorbance coefficient 6.2 mM^{-1} cm^{-1}. One unit is the amount of enzyme that oxidizes 1 nmol of NADPH per min at 25°C (Foyer and Halliwell 1976).

Determination of glutathione levels

Oxidized (GSSG) and reduced (GSH) glutathione levels were determined by following the formation of

thio-nitrobenzoic acid (TNB) via the recycling assay with glutathione reductase. Total glutathione content (GSH+2GSSG) was measured by adding either standards (GSH) or properly diluted samples to the reaction mixture containing 0.1 M phosphate buffer (pH 7.4), 6.3 mM EDTA, 0.73 mM 5,5-dithio-bis-nitrobenzoic acid (DTNB), 0.24 mM NADPH, 0.09% 5-sulfosalicylic acid (SSA), and 1.2 U of glutathione reductase (GR) in a final volume of 1 ml. After incubating for 20 min at room temperature, the assay was measured at an absorbance of 415 nm. An additional reaction allowed the determination of GSSG alone. In this reaction, any GSH present in the sample was first removed by vortexing for 1 h at room temperature with 0.05% of 2-vinylpyridine. After centrifugation, GSSG was measured by the same method for total glutathione measurement. The differences between the total glutathione equivalents (GSH+2GSSG) and twice the measured GSSG alone, was determined to be the measured amount of GSH in the sample (Gaullier et al. 1994).

Two-dimensional gel electrophoresis and MALDI-TOF MS analysis

Yeast cells were grown to the mid-log phase and exposed to hydrogen peroxide for 1 h at 30°C with shaking. Cells were harvested by centrifugation, washed twice with cold-PBS buffer, suspended in lysis buffer containing 50 mM Tris–HCl, pH 7.5, 2% SDS, 5% glycerol, 2% β-mercaptoethanol (ME), 2 mM EDTA and 1 mM PMSF, added to an equal of glass beads (425–600 μm; Sigma, USA), and disrupted with MicroMixer. To inactivate protease activity, cells were boiled for 5 min and cooled on ice for 5 min. Protein crude extracts were collected by high speed centrifugation (13,000 rpm, 4°C, 10 min), and incubated with DNase/RNase/Mg mixture on ice for 15 min. Samples were precipitated by 10% TCA on ice for 1 h and centrifuged at 13,000 rpm for 10 min at 4°C. The pellets were washed three times with cold-ethanol containing 0.1% ME, vaccum-dried, resuspened in sample buffer containing 9.5 M urea, 4% CHAPS, 40 mM Tris, 0.1 M DTT and 0.2% Bio-Lyte (3-10; Bio-Rad) at room temperature with shaking, and centrifuged at 15,000 rpm for 30 min. The cleared supernatants were carefully collected. 0.75 mg of proteins were loaded onto preparative pH 4–7 IPG strips (17 cm, linear; Bio-Rad) and rehydrated for 16 h at 20°C at passive condition. Focusing conditions are as follows: 250 V for 1 h, 250–10,000 V for 5 h, and 10,000 V for 9 h. After isoelectric focusing (IEF), the gel strips were equilibrated. SDS-PAGE was done with 12% gels, run at 10 mA per gel for the first 1 h and followed by 25 mA per gel, stained with Coomassie Brilliant Blue R-250 (CBB R-250), and then destained. Spots with significant changes were considered to be accumulated proteins. Interesting spots were excised from the gel. In-gel digestion and MALDI-TOF MS analysis was done by Genomine Inc. (Korea). Protein identification was analyzed using MASCOT program (http://www.Matrixscience.com).

Redox state analysis

Cells were cultured at 30°C until reaching mid-log phase and then exposed to 30 mM H_2O_2 for 1 h. The cells were harvested and lysed with glass beads. The intracellular hydroperoxide level was determined by ferrous ion oxidation in the presence of a ferric ion indicator, xylenol orange (Deiana et al. 1999). Fifty μl of crude cell extract were added to 950 μl of FOX reagent [100 μM xylenol orange (water-soluble form; Sigma, USA), 250 μM of ammonium ferrous sulfate, 100 mM of sorbitol, and 25 mM of sulfuric acid]. The mixture was incubated at room temperature for 30 min and then, centrifuged to remove any flocculants before measuring the absorbance at 560 nm. The hydroperoxide concentration was represented by nmol per mg protein. To measure cytosolic ROS, exponential cells were incubated for 20 min at 30°C with 50 μM dichlorodihydrofluorescein diacetate (DCFHDA; Invitrogen, USA), exposed to 30 mM H_2O_2 for 1 h, washed twice with PBS buffer, and then resuspended in the same PBS buffer. Cells loaded with the fluorescent probes were imaged with a fluorescence microscopy (excitation, 488 nm; emission, 525 nm). Protein oxidation was detected by immunoblot. Briefly, lysates were prepared from mid-log phase cells exposed to 30 mM H_2O_2 for 1 h with shaking (160 rpm). 30 μg of protein from each were reacted with 2,4-dinitrophenylhydrazine (DNPH) for 15 min at 25°C. Samples were resolved on 12% denaturing polyacrylamide gels, and DNP-derivatized proteins were analyzed using an anti-DNP (dinitrophenol) antibody (Sigma, USA). Lipid peroxidation was analyzed by western blot using anti-MDA antibody (SantaCruz, USA).

Laboratory-scale batch fermentation

To compare fermentation ability, the cells were grown anaerobic fermentation conditions on shaker (160 rpm) for 2.5 days at 30°C in YG medium containing 20% (w/v) glucose and 1% (w/v) yeast extract. Alcohol concentration was determined from the percentage (v/v) of alcohol in the distillate after fermentation, using the alcohol hydrometer REF 503 (Korins, Korea). Residual total glucose concentration was measured using a Hand-Held Refractometer N1 (Atago, Tokyo). Samples were obtained after removing cell debris by centrifugation. To monitor the growth kinetics during the fermentation process, the cells were grown in YG medium and their optical density was measured at 600 nm at 2-h intervals for 16 h.

Table 1 Strains used in this study

Strain	Genotype	Source
E. coli		
DH5α	*endA, RedA, hsd, deoR, LacZ M15*	KCTC
S. cerevisiae		
BY4741 (BY)	*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0*	EUROSCARF
WT	*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0*, pVTU260	This study
TG	*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0*, BrGR::pVTU260	This study

EUROSCARF European Saccharomyces Cerevisiae Archive for Functional Analysis; *KCTC* Korean Collection for Type Cultures

Results

Construction and expression of BrGR recombinant yeast

In the presence 30 mM H_2O_2, the cell viability and GR activity of yeast cells decreased by approximately 40% compared to yeast cells without 30 mM H_2O_2 (Fig. 1a). Based this result, we examined whether GR overexpression could have an effect on acquired tolerance to oxidative stress in yeast cells since yeast GR expression is down-regulated in the presence of hydrogen peroxide. A cDNA containing the open reading frame (ORF) region of cytosolic GR from *B. rapa* subsp. *pekinensis* was cloned into an yeast expression vector pVTU260 that allows constitutive expression of an genes of interest under the control of the yeast *ADH1* promoter (Fig. 1b). After digestion with the restriction enzyme *Nco*I and *BamH*I, the DNA fragment was approximately 1.51 kb in size. The construct was sequenced to confirm the correct ORF of the insert included the coding region of GR to verify that no nucleotides were deleted, inserted, or substituted during the cloning, which would alter the amino acid sequence during the translation of the cloned gene, and finally, the plasmid was introduced into the *S. cerevisiae* BY4741 strain. The complete BrGR ORF encodes a 503-amino-acid protein, with a predicted molecular weight of approximately 55 kDa. The deduced amino acid (A.A) sequences were

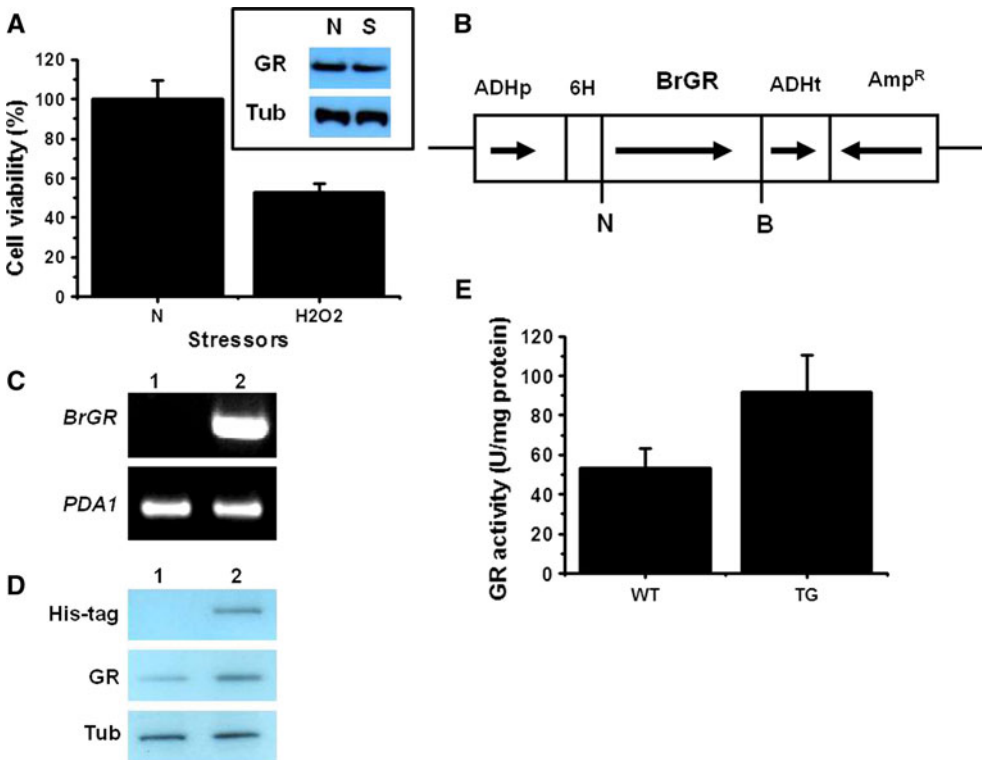

Fig. 1 Construction of BrGR expression vector and protein expression in yeast. **a** Cell viability and glutathione reductase expression. Cell viability was represented to be 100% of BY4741 cells without 30 mM H_2O_2 (N) for 1 h. Glutathione reductase expression was analyzed using Western blot (*boxed*). **b** Schematic diagram of BrGR expression vector. Approximately 1.5 kbp GR gene with *Nco*I (N) and *Bam*HI (B) restriction enzyme sites was subcloned downstream to the ADH promoter generating the recombinant BrGR::pVTU260 expression vector. *ADHp* alcohol dehydrogenase (ADH) promoter; *6H* six histidine-tagged resides; *ADHt* ADH terminator; *Amp*^{R} ampicillin resistance gene. To examine whether BrGR is expressed in the yeast system, semi-quantitative RT-PCR (**c**), Western blot (**d**) and enzyme assay (**e**) were done. PCR amplicon of *PDA* gene and anti-tubulin (*Tub*) antibody were used for a housekeeping control in RT-PCR and immunoblot analysis, respectively. Enzyme activity was represented as specific activity (U/mg protein). *1* WT cells; *2* TG cells

92.2%, and 79.5% identical to those of *Arabidopsis thaliana* (Accession number: P48641), and *Pisum sativum* (garden pea; Q43261), respectively (Lee et al. 1998). The A.A sequences were 34.7% identical and 52% similar to those of *S. cerevisiae* (NP_015234). It exhibited a high degree of conservation within the region responsible for the pyridine nucleotide-disulphide oxidoreductase active site and for the binding of FAD, GSSG, or NAD(P)H (data not shown) (Lee et al. 1998). To explore whether the *BrGR* gene is effectively expressed in yeast, we did RT-PCR analysis, under the conditions described in the "Materials and methods". One DNA fragment of 951 bp corresponding to the region between the *BrGR* ORF was amplified in BrGR-expressing transgenic yeast cells (TG cells), while no amplification signal was detected in a wild-type cells with the vector alone (WT cells) (Fig. 1c). To determine whether the *BrGR* gene is properly expressed at the translational level, we also did immunoblotting and an enzyme assay. As shown in Fig. 1d, we confirmed the ectopic expression of BrGR in the TG cells, but not the WT cells because the endogenous yeast GR protein in the WT cells was non-reactive to anti-His tag antibody even though anti-GR antibody was detected in both TG and WT cells. Ectopic BrGR expression was strongly supported by the evidence from the enzyme assay. Total GR activity was two fold higher in TG cells than in WT cells grown under normal conditions (Fig. 1e). These results indicate that ectopic expression of BrGR could play an important role in yeast upon abiotic conditions because BrGR exhibits a high degree of conservation within the region responsible for the redox reaction including the residues important in binding GSSG, the redox-active disulfide bridge region, and the two conserved arginine residues required for binding of the 2′-phosphate group of NADPH (Fig. S1).

Stress tolerance to hydrogen peroxide in BrGR-expressing transgenic yeast cells

The effect of oxidative challenge on growth rate and survivorship was evaluated in yeast cells transformed with the pVTU260 plasmid with either GR gene or just the vector, for a series of experiments, exploring the ability of BrGR expression to enhance resistance to oxidative stress in *S. cerevisiae*. The pVTU260 plasmid is a vector that expresses constitutively under the ADH promoter. H_2O_2 at a toxic concentration of 5.0 mM has been used to investigate the oxidative stress response in *S. cerevisiae* (Toledano et al. 2003). Growth kinetics of the TG yeast cells was higher than that of the WT cells when the cells were cultured on liquid medium in the presence of 3.5 mM H_2O_2. For the first 8 h, growth of the TG yeast cells occurred at almost the same rate as that in the WT yeast cells. A lag then ensued, with growth resuming, but more rapidly. The TG yeast cells entered the stationary phase at 18 h. The WT yeast cells had a longer lag phase period (12 h) compared to a rapid restoration of growth kinetics in the TG yeast cells. The appearance of the stationary phase was achieved at about 22 h after initial inoculation (Fig. 2a). There was no difference in the growth rates between the TG yeast cells and the WT yeast cells under normal conditions (data not shown). Stress tolerance was also detected using the spotting assay with serial dilutions. Stress acquisition of the TG yeast cells exposed to 30 mM H_2O_2 for 1 h was higher than that of the WT yeast cells (Fig. 2b). To check if the acquired tolerance of the TG yeast cells to oxidative stress resulted in GSH regeneration by BrGR, the cellular GSH content was estimated. The glutathione level of both mid-log phase WT and TG yeast cells was analyzed in YPD-grown cultures. Changes in total glutathione (tGSH), reduced glutathione (GSH), and oxidized glutathione (GSSG) was observed in both cell lines. The tGSH level was markedly increased after treatment with 30 mM H_2O_2. The amplitude of these variations was higher for the TG yeast cells than for the WT yeast cells. BrGR-expressing transgenic yeast cells showed increased GSH and decreased GSSG concentrations against H_2O_2-induced oxidative stress as compared to the wild-type yeast cells (Table 2), which led to a higher increase in the GSH to GSSG ratio in the TG yeast cells compared to the WT yeast cells under the same conditions (Fig. 2c). The high GSH accumulation in the TG yeast cells was important for it to adapt and/or overcome unfavorable condition because *gsh1* and *gsh2* mutant cells (EUROSCARF) involved in glutathione synthesis and *glr1* mutant cells (EUROSCARF) encoding glutathione reductase were hypersensitive to H_2O_2-induced oxidative stress when compared to wild-type yeast cells (Fig. 2d). Therefore, these results suggest that the ectopic BrGR expression in TG yeast cells enhances and improves the tolerance via GSH accumulation by GSH reproduction using BrGR against the oxidative stress environment.

Co-activation of cell rescue proteins and the redox state in BrGR-expressing yeast cells

Oxidant-induced protective responses often result from the coordinated activation of proteins involved in oxidant neutralization. Because cellular antioxidants act in a concerted manner, we investigated whether the modulation of ectopic BrGR expression caused changes in the activity of other major cell rescue proteins. The experiment was done using exponential phase yeast cells in the presence of H_2O_2 and analyzed by 2-DE and immunoblotting. As seen in Fig. 3a, b and Table 3, the expression of proteins involved in metabolic systems via glycolysis containing hexokinase (Hxt), fructose-1,6-biphosphate aldolase (Fba1p), glyceraldehydes-3-phosphate dehydrogenase (GAPDH), enolase

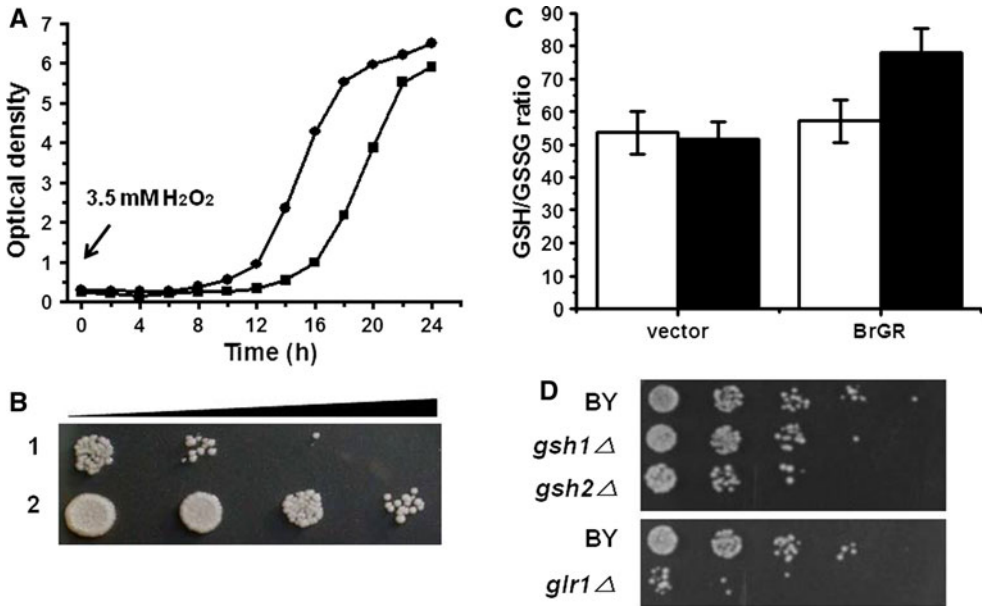

Fig. 2 A stress tolerance and sensitivity assay, and ratio of GSH to GSSG and stress sensitivity of yeast cells under hydrogen peroxide. **a** The growth rate was spectrophotometrically monitored at 600 nm for 24 h in the presence of 3.5 mM H_2O_2. **b** For spotting assays, mid-log phase cells were exposed to 30 mM of hydrogen peroxide for 1 h. A tenfold series dilution was done and 5 µl of each dilution was spotted onto an YPD agar plate. *Square* or *1* WT cells; *circle* or *2* TG cells. **c** Ratio of reduced form (GSH) to oxidized form (GSSG) in the absence (*white bar*) and presence (*black bar*) of 30 mM H_2O_2 for 1 h. **d** Stress sensitivity of gene-deficiency yeast cells involved in glutathione system in the presence of 20 mM H_2O_2 for 1 h. BY, *S. cerevisiae* BY4741 cells

Table 2 Glutathione level under hydrogen peroxide stress

	tGSH	GSSG	GSH
Without H_2O_2			
WT	171.98 ± 25.58	3.09 ± 0.17	165.78 ± 25.23
TG	201.74 ± 30.16	3.41 ± 0.14	194.91 ± 29.87
With H_2O_2			
WT	237.16 ± 45.24	4.41 ± 0.18	228.32 ± 44.88
TG	303.96 ± 40.23	3.84 ± 0.17	296.27 ± 39.88

The contents were represented as a nmol/10^9 cells/ml

tGSH total glutathione; *GSSG* oxidized form; *GSH* reduced form

(Eno2p), 3-phosphoglycerate kinase (Pgk1p), enolase (Eno2p) and aldehyde dehydrogenase (Ald), pentose phosphate pathway (glucose-6-phosphate dehydrogenase, G6PDH) involved in reducing power (NADPH), antioxidant systems containing glutathione peroxidase (Gpx), thioredoxin (Trx2 and Trx3) and thioredoxin reductase (Trr1), molecular chaperone and its cofactors containing Hsp104, Hsp90, Hsp42 Hsp26 Cpr1p, Grp, Sti and Zpr1, and zinc-finger protein of unknown function (Stp3p) was up-regulated in the TG yeast cells under oxidative stress compared to the WT yeast cells. Western blot analysis clearly supported MALDI-TOF MS data following 2-DE. The expression of Ssa, Ssb, thioredoxin peroxidase (Tsa1) and Hsp60 remained unchanged or reduced in the yeast cells in the presence of H_2O_2. BrGR expression in TG yeast cells can lead to increased energy-generating proteins (NADPH and ATP) and protein homeostasis following the elevation of chaperones with protein folding abilities under oxidative stress. In contrast, the difficulty of maintaining redox homeostasis in the WT yeast cells could make it vulnerable to oxidative stress caused by H_2O_2. The breakdown of cellular redox balance involves the modulation of cellular oxidative biomarkers such as ROS level and an increase in carbonyl content due to protein oxidation. The pattern changes in protein carbonylation and lipid oxidation were analyzed with western blot by using anti-dinitrophenyl (DNP) and anti-malondialdehyde (MDA), respectively. Protein carbonylation was clearly higher in the WT yeast cells than the TG yeast cells during oxidative stress (Fig. 3c, left), whereas MDA levels remained unchanged in both types of cells under the same conditions because of lipid peroxidation (Fig. 3c, right). One of the important strategies when yeast cells are exposed to an oxidative environment is protein decarbonylation because carbonylation is an irreversible and irreparable modification although it has been noted that carbonylated proteins can be more sensitive to proteolytic degradation than nonoxidized proteins. To investigate whether increased protein carbonylation in the WT cells is involved in the cellular redox state, the cellular ROS level was measured in the absence and presence of H_2O_2. Hydroperoxide level using FOX reagent in the TG yeast cells was approximately 1.4-fold lower than that in the WT yeast cells during H_2O_2

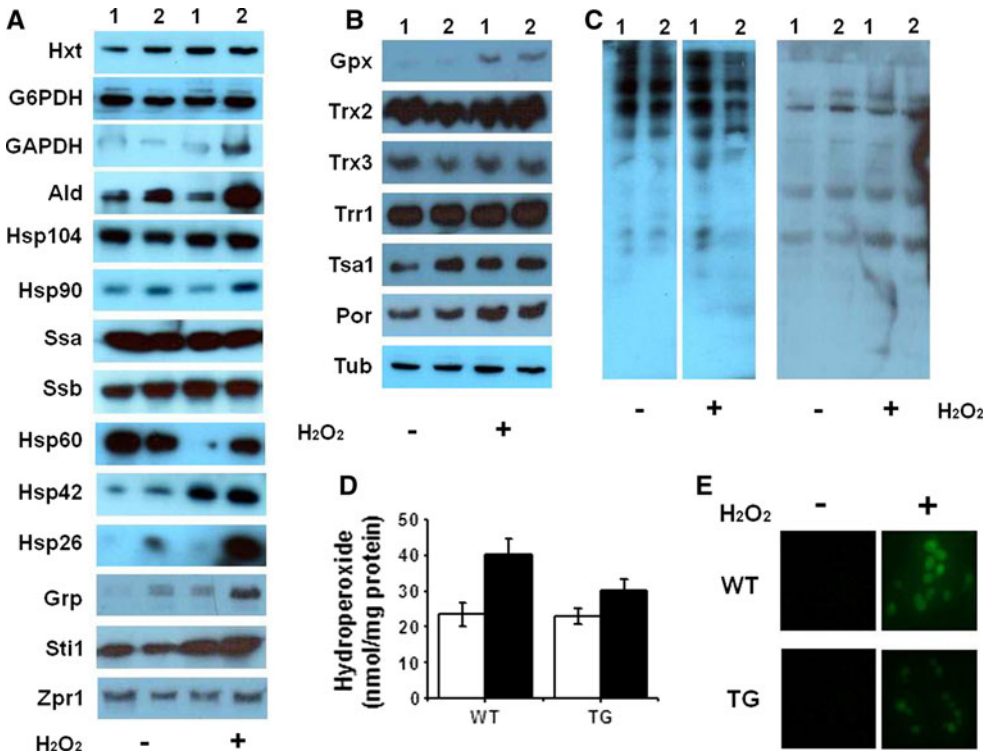

Fig. 3 Cell rescue proteins' expression and redox state under oxidative condition. Western-blot analysis of metabolic enzymes and molecular chaperones (**a**), and antioxidative proteins (**b**) without (−) and with 30 mM H_2O_2 (+) for 1 h. Anti-tubulin (*Tub*) antibody was used for a standard control. **c** Protein carbonylation (*left*) and lipid peroxidation (*right*) analysis using anti-DNP and anti-MDA antibodies in the absence (−) and presence of 30 mM H_2O_2 (+) for 1 h. 30 μg of crude extract protein was loaded onto 12% SDS-PAGE gel. *1* WT cells; *2* TG cells. Redox state analysis using FOX reagent (**d**) and oxidant-sensitive probe DCFHDA (**e**) when mid-log yeast cells were exposed to 30 mM H_2O_2 for 1 h

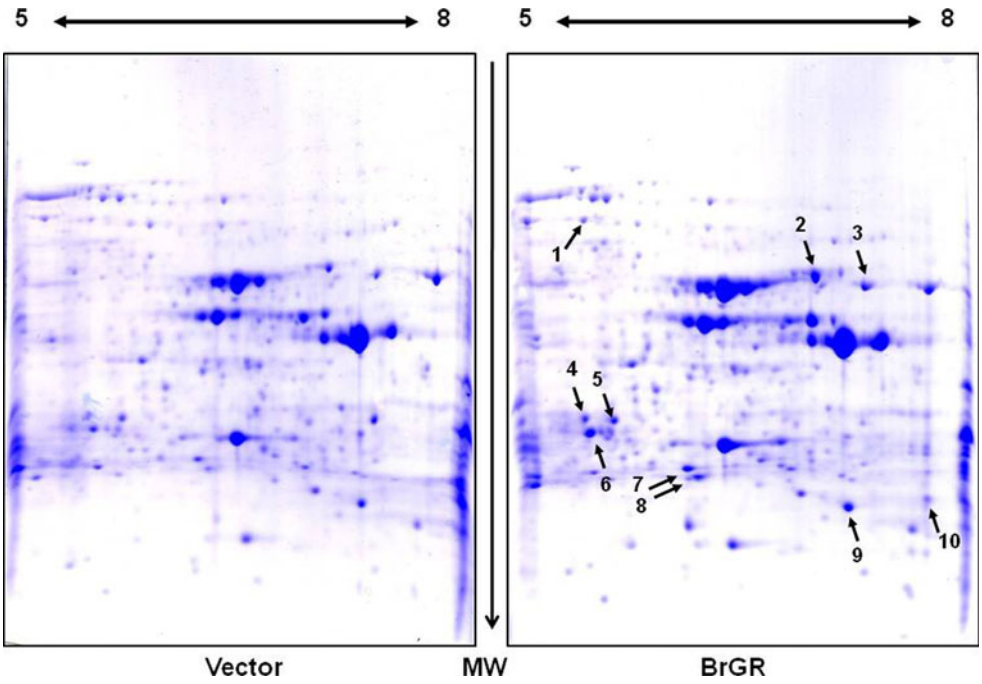

Fig. 4 A comparative analysis by 2-D gel electrophoresis of whole cellular proteins between the wild type and BrGR-expressing strains of *S. cerevisiae* under hydrogen peroxide stress. Total soluble protein samples were obtained from exponentially-growing cells, which were treated with 30 mM of H_2O_2 for 1 h. After rehydration, the first dimension was performed on a ready-made IPG strip (pH 4-7, 17 cm) and sample application (0.75 mg protein). The running conditions were same as described in "Materials and methods". After SDS-PAGE (12%) and CBB staining, interest spots were cut. After digestion with trypsin, MALDI-TOF MS analysis was done

management even though the hydroperoxide concentration in the TG yeast cells increased in the presence of H_2O_2 (Fig. 3d). To confirm these findings, the levels of intracellular hydroperoxides were evaluated by fluorescence microscopy with a cytosolic oxidant-sensitive probe DCFHDA. An increase in DCF fluorescence was observed

Table 3 Proteins identified by MALDI-TOF MS analysis

Spot No.	Protein information	MW (Da)	pI	Cov. (%)	Score
1	Wtm1p	48,467	5.18	29	157
2	Chain A, catalytic metal ion binding in enolase: crystal structure of enolase-Mn2+-phosphoacetohydroxamate complex	46,558	6.04	50	257
3	Phosphoglycerate kinase (Pgkp)	44,595	7.71	50	226
4	Eno2p	46,942	5.67	43	150
5	Eno2p	46,942	5.67	34	144
6	Hsp26p	23,865	5.31	44	129
7	YKL060Cp (Fba1p)-like protein	24,700	4.98	35	79
8	Fba1p	39,881	5.51	35	105
9	Cpr1p	17,494	6.90	39	62

in both WT and TG yeast cells when they were exposed to H_2O_2. The intensity in fluorescence was more pronounced in the WT yeast cells as compared to the TG yeast cells. In addition, we observed a moderate release of the probe outside the cells in the WT yeast cells (Fig. 3e). Increased ROS levels are in agreement with increased oxidative protein damage following a reduction of cell rescue proteins in the WT yeast cells (Figs. 3, 4). To sum up our results, the alleviated redox state is supported by the results from cell viability, ROS level, and antioxidative proteins in the TG yeast cells under oxidative stress.

A marked effect on stress tolerance in an ectopic BrGR-overexpressing strain under diverse conditions

Because GR expression remained unchanged or decreased in WT cells under environmental conditions (Fig. 5), we also performed assays to determine whether BrGR over-expression could improve acquired tolerance to different

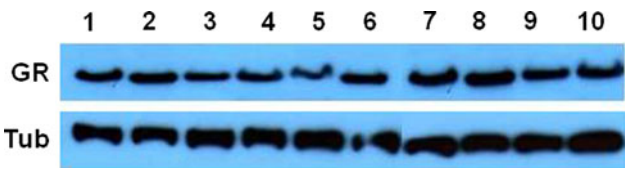

Fig. 5 GR expression analysis in wild-type cells under different stress conditions. Mid-log phase cells were exposed to exogenous stimuli for 1 h at 30°C with shaking. Protein extraction, SDS-PAGE (12%) and immunoblotting analyses were performed as described in "Materials and methods". *1* non-stressed cells; *2* heat shock; *3* 30 mM H_2O_2; *4* 0.2 mM menadione; *5* 10 mM t-BOOH; *6* 1.0% SDS; *7* 5.0 mM $CdCl_2$; *8* 10 mM $CuSO_4$; *9* 0.1 M $ZnCl_2$; and *10* 5.0 M NaCl-treated cells. For heat shock, the cells were challenged for 10 min at 55°C. Anti-tubulin antibody was used as a housekeeping control

kinds of stressors such as oxidants (menadione and *t*-BOOH), heavy metals (cadmium, zinc, and copper), the detergent sodium dodecyl sulfate (SDS), high salinity (i.e., NaCl level), ethanol, and heat shock in yeast cells. A comparative analysis of stress tolerance was done using growth kinetics and the spotting assay. Cell survival of TG yeast cells constitutively expressing the BrGR protein under regulation of the ADH promoter was observed in serial dilutions when yeast cells were exposed to 0.2 mM menadione (MD), 10 mM *t*-BOOH, 1.0% SDS, 5 mM cadmium chloride, 0.1 M zinc chloride, 20 mM copper sulfate, and 5.0 M NaCl, respectively, for 1 h. The cells were heat shock at 55°C for 20 min. Recovery ability of the TG yeast cells was more rapid than that of the WT yeast cells in the presence of MD, *t*-BOOH, SDS, cadmium (Fig. 6, boxed photograph), zinc, copper, NaCl, and heat shock (Fig. 7, boxed photograph). A higher stress response in the TG yeast cells was also detected by the growth rate. Growth kinetics was analyzed by measuring changes in the optical density at 600 nm in YPD medium supplemented with 40 μM MD, 1.0 mM *t*-BOOH, 0.2% SDS, 50 μM $CdCl_2$, 10 mm $ZnCl_2$, 5 mM $CuSO_4$, and 1.0 M NaCl, respectively. As shown in Figs. 6, 7, there was a significant difference in the growth kinetics. For early phase, growth of the TG yeast cells occurred at almost the same rate as that of the WT yeast cells. A temporary lag then ensued, with growth resuming, but more rapidly. The final A_{600} in the TG yeast cells was considerably higher than that in the WT yeast cells during the indicated time. In addition, BrGR-expressing TG cells were found to be more resistant than WT cells against high ethanol concentrations, which depended on high ethanol concentration (Fig. 8). Ectopic expression of BrGR dramatically created rapid restoration in the presence of stressors. Thus, these results show that BrGR-expression in TG yeast cells could enhance the acquisition tolerance to pro-oxidants, heavy metals, SDS, high salinity, and alcohol compared to the WT yeast cells.

Effect of BrGR over-expression under batch fermentation conditions

Because BrGR-expressing TG cells were more resistant than WT cells to high ethanol concentrations as well as exogenous stimuli, the laboratory-scale fermentations involving TG and WT yeast cells were performed at 30°C. No significant differences were observed between TG and WT cells with respect to the alcohol yield and residual glucose content at 30°C, which is the general temperature generally used for industrial fermentation (Fig. 9a, b). During fermentation at 30°C, the growth kinetics of TG cells slightly decreased when they were grown in YG medium; however, the growth kinetics of both types of cells did not significantly differ when they were grown in

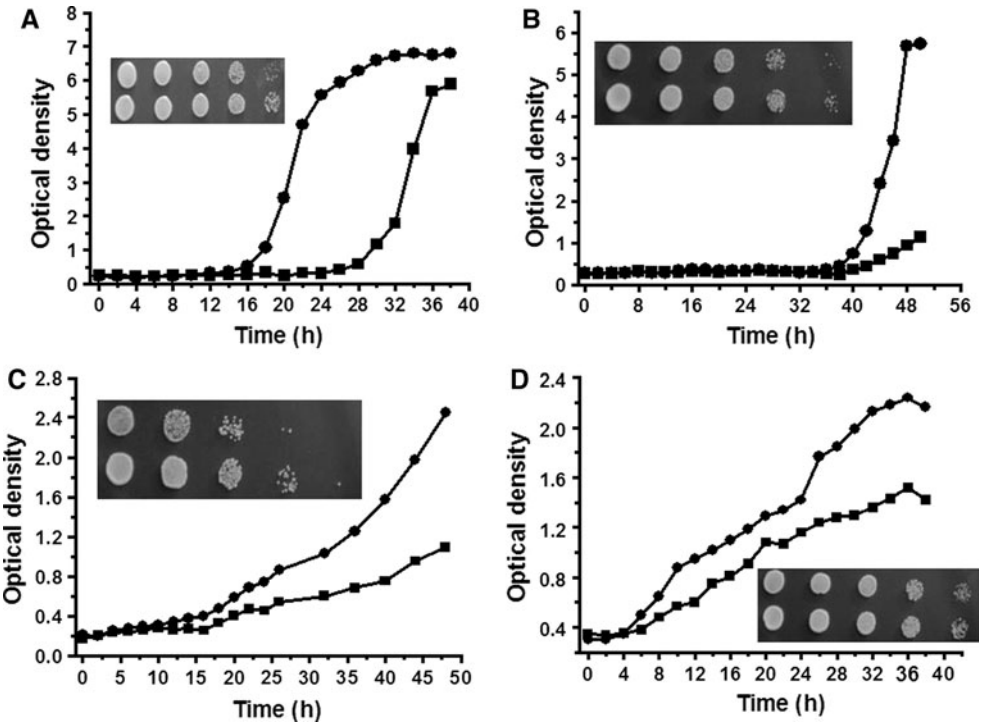

Fig. 6 Stress tolerance of BrGR-expressing yeast cells against menadione, t-BOOH, SDS, and cadmium. The growth rate of the yeast cells was monitored at different times in the presence of 40 μM menadione (**a**), 1.0 mM t-BOOH (**b**), 0.2% SDS (**c**), 0.4% SDS, and 50 μM $CdCl_2$ (**d**), respectively. For spotting assays, once reaching the mid-log phase ($A_{600} = 3.5$), the cells were exposed to 0.2 mM menadione (**a**, *boxed*), 10 mM t-BOOH (**b**, *boxed*), 1% SDS (**c**, *boxed*), and 5 mM $CdCl_2$ (**d**, *boxed*) for 1 h, diluted properly, and then spotted onto YPD agar plate. These results were presented inside growth kinetics data. *Square* or *upper panel* WT cells; *circle* or *lower panel* TG cells

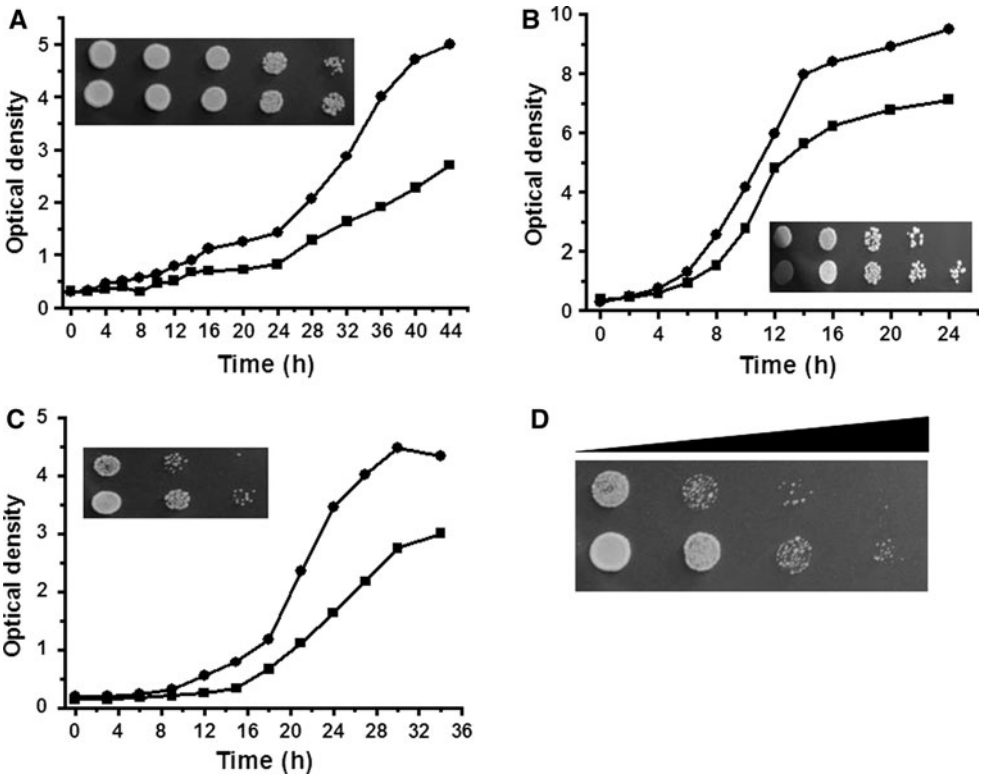

Fig. 7 Stress response of BrGR-expressing transgenic yeast cells to zinc, copper, high salinity, and heat shock. The growth kinetics of the yeast cells was measured at different times in the presence of 10 mM $ZnCl_2$ (**a**), 5 mM $CuSO_4$ (**b**), and 1 M NaCl (**c**), respectively. For spotting assays, mid-log phase cells ($A_{600} = 3.5$) were exposed to 0.1 M $ZnCl_2$ (**a**, *boxed*), 20 mM $CuSO_4$ (**b**, *boxed*), and 5 M NaCl (**c**, *boxed*) for 1 h, respectively, with shaking. Heat shock was treated for 20 min at 55°C (**d**). After stress challenge, yeast cells were properly diluted, and then spotted onto YPD agar plate. *Square* or *upper panel* WT cells; *circle* or *lower panel* TG cells

YPD medium (Fig. 9c). The spotting assay results did not significantly differ between TG and WT cells in the presence of high glucose concentrations (Fig. 9d). GR activity was measured to check whether high glucose concentrations affect BrGR activity. GR activity in TG cells decreased gradually during the fermentation process and was lower than in WT cells (Fig. 9e). These results indicate that a high glucose concentration affects GR

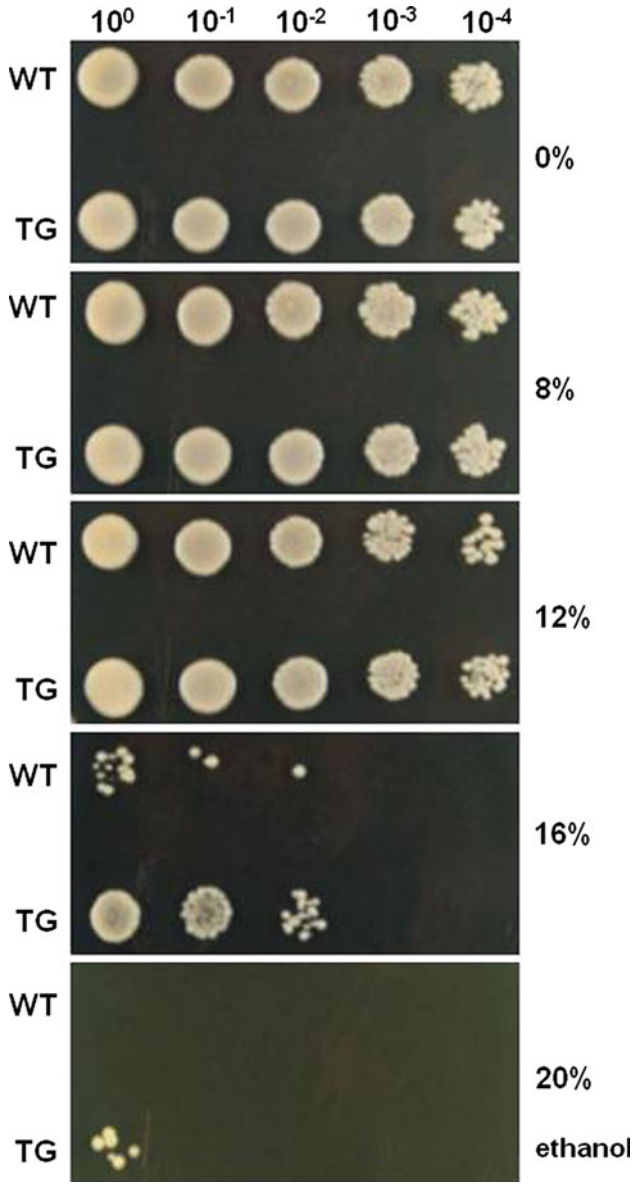

Fig. 8 Ethanol tolerance assay. Mid-log phase yeast cells were exposed to ethanol (0, 8, 12, 16 and 20%) for 1 h, diluted serially from 10^0 to 10^{-4}, and were then spotted onto YPD agar plates. *WT* wild-type cells with vector alone; *TG* BrGR-expressing yeast cells

activity, especially BrGR activity, which is a limiting factor for the corresponding calculated fermentation yield during fermentation in TG cells.

Discussion

Stress containing oxidative stress is caused during respiratory process for energy production or by exogenous stimuli, which can alter the intracellular environment, allowing the cellular redox state to become more oxidized. Aerobic organisms have developed a set of cell rescue systems to minimize or prevent the detrimental effects to stress. Among these defense systems, it has been proposed elsewhere that glutathione reductase (GR) participates in cell protection against stress conditions, such as oxidative stress by maintaining the adequate redox balance in the intracellular environment and thus, regulating various cellular activities in different organisms (Jamieson 1998; Costa and Moradas-Ferreira 2001). In this study, we have shown that ectopic BrGR-expression in the transgenic yeast cells is very specific in the protection of cells against ROS-induced oxidative stress including pro-oxidants (menadione and t-BOOH), heavy metals (cadmium, copper and zinc), SDS, high salinity and heat shock (Figs. 6, 7), especially H_2O_2 (Fig. 2). The protective action of BrGR was demonstrated by growth kinetics, cell viability, and changes in cell rescue proteins in the TG yeast cells when yeast cells were exposed to H_2O_2. ROS levels in WT yeast cells with just the vector alone were higher than those in the BrGR-expressing transgenic yeast cells (Fig. 3d, e). ROS production can affect cellular components and increase oxidative biomarker, protein oxidation (protein carbonylation), but not lipid peroxidation (Fig. 3c). To overcome or prevent overwhelming production of ROS, cells have many antioxidative mechanisms to detoxify ROS. Glutathione (GSH) is a very well known non-enzymatic antioxidant molecule. GSH concentrations in the TG yeast cells was higher than in the WT yeast cells under oxidative condition (Table 2), which led to an increase in the GSH/GSSG ratio (Fig. 2c). The high GSH accumulation in the TG yeast cells is a result of GSH regeneration by BrGR expression because the GSH content and GSH/GSSG ratio was lower in WT yeast cells under H_2O_2-induced oxidative stress. The GSH, a non-protein thiol compound abundant in almost all aerobic organisms, is essential for the resistance and adaptation to hydrogen peroxide in yeast cells, because mutant strains (*gsh1* and *gsh2*) encoding glutathione synthesis in the presence of ATP and mutant strain (*glr1*) encoding glutathione rerecycling was hypersensitive to hydrogen peroxide (Fig. 2d) (Flattery-O'Brien and Dawes 1998). As shown in our results, previous results showed that yeast strains lacking GSH or altered in their GSH redox state are sensitive to oxidative stress induced by peroxides and superoxide anions, as well as toxic compounds from lipid peroxidation (Grant 2001). Exponential phase cells grown on rich YPD medium under normal aerobic conditions have a high redox ratio (GSH/GSSG) of 11–16:1, indicating that most of the GSH is maintained in a reduced form (GSH) (Table 2). Unlike the TG yeast cells, exposure of the WT yeast cells to H_2O_2 causes a reduction in the GSH/GSSG ratio and GSH levels (Table 2), as well as a shift in the redox balance following increases in the levels of protein mixed disulfides between cysteine residues and low

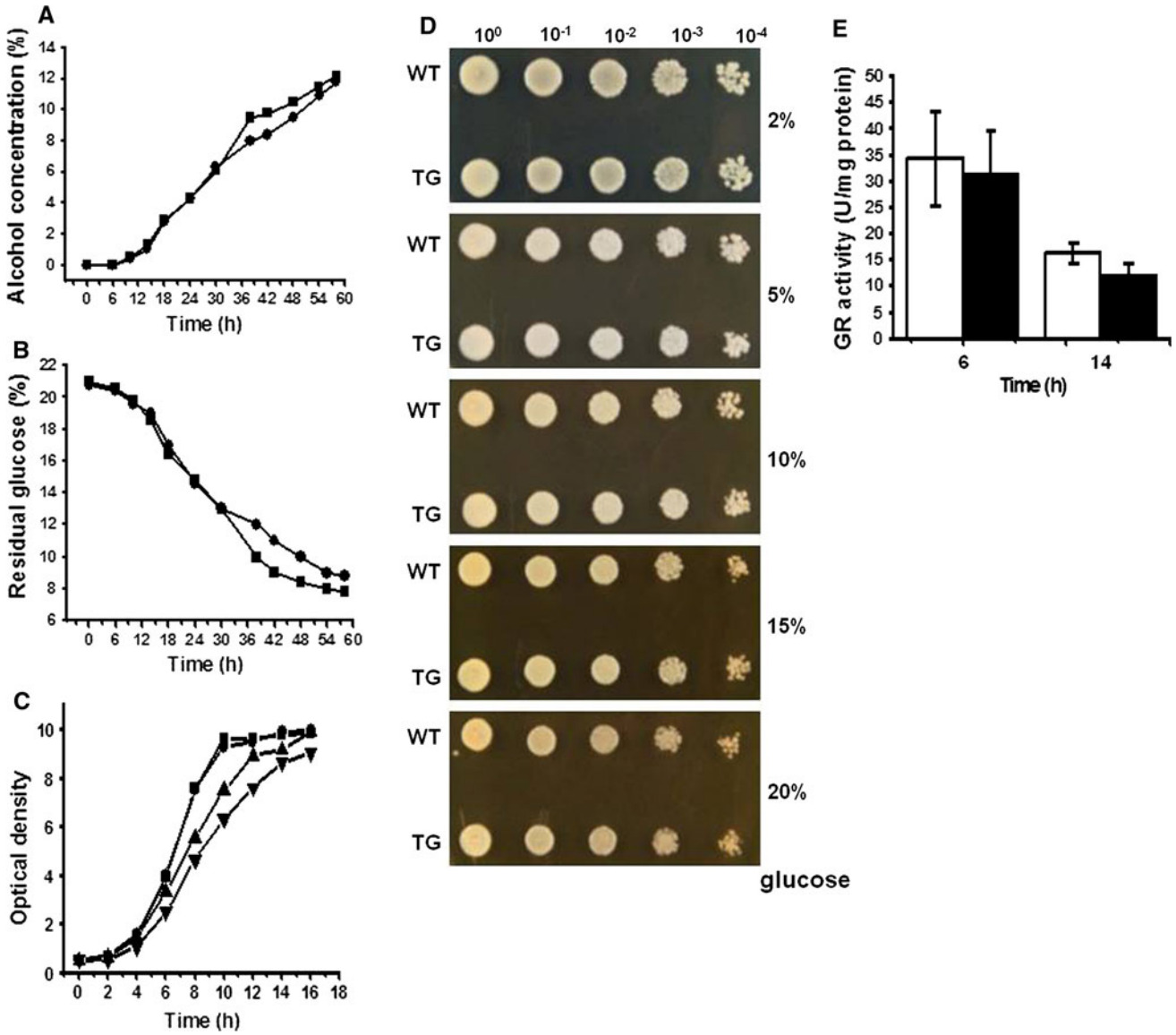

Fig. 9 Fermentation ability, growth kinetics and GR activity during the fermentation process. Fermentation ability was analyzed by measuring the alcohol (**a**) and residual glucose (**b**) concentration in YG medium. *Square* WT cells; *circle* TG cells. **c** Growth kinetics was determined by measuring the optical density at 600 nm. *Square* WT cells in YPD medium; *circle* TG cells in YPD medium; *triangle* WT cells in YG medium; *reverse triangle* TG cells in YG medium. **d** The high glucose adaptation levels of yeast cells were analyzed using a spotting assay. *WT* wild-type cells with vector alone; *TG* BrGR-expressing yeast cells. **e** GR activity was measured by sampling at 6 and 14 h after initiating fermentation. *White bar* WT cells; *black bar* TG cells

molecular thiols (protein-S-thiolation) (Grant et al. 1998). GSSG levels in the TG yeast cells were 1.2-fold higher under oxidative conditions compared to normal conditions even though the level was lower (1.2-fold) in TG yeast cells compared to the WT yeast cells. Therefore, increase of GSH contents by BrGR could enhance and improve the redox state upon H_2O_2 exposure in TG yeast cells.

The activity of GR is often analyzed together with the activities of other defense enzymes for a complete understanding of the cellular antioxidant profile under physiological or oxidative stress conditions. The BrGR-expressing transgenic yeast cells also induced a wide range of enzymatic cell rescue proteins containing metabolic enzymes (Hxt, Fba1, Pgk, G6PDH, GAPDH and Ald) (Fig. 3a) and antioxidant systems (Gpx, Trx2, Trx3, Trr1) (Fig. 3b) under H_2O_2 challenge compared to the wild-type yeast cells. Repression or down-regulation of proteins involved in glycolytic metabolism and the non-oxidative branch of the pentose phosphate pathway that are important for ATP and NADPH, such as GAPDH and G6PDH, increases stress sensitivity to oxidant and also are defective in adaptation to oxidative stress (Tan et al. 2009).

Glutathione-dependent peroxidases (Gpx) and thioredoxin-dependent peroxidases (Tsa1 etc.) convert hydrogen peroxide to water and oxygen. In GSH- and TRX systems, oxidized glutathione (GSSG) and thioredoxin (oTrx), are reduced by glutathione reductase (GR) and thioredoxin reductase (Trr), respectively. Hexokinase (Hxk1), Trx2, thioredoxin peroxidase (Tsa1, Tsa2 and Prx1), glutathione precursor synthesis (Ecm38) and glutathione peroxidase (Gpx1, Hyr1) are involved in the stress response to oxidative stress (Gasch et al. 2000; Tucker and Fields 2004). Deletion or downregulation of these antioxidant agents lead to a number of oxygen-dependent phenotypes, including oxygen sensitivity, slow growth, hypersensitivity to oxidants, which are believed to accelerate aging, and auxotrophy for methione and lysine (Tan et al. 2009). Cellular NADPH that functions as a reducing power for GR and Trr is important for tolerance to ROS. Deleted or repressed genes involved in the nonoxidative branch of the pentose phosphate pathway that are important for NADPH production, such as G6PDH (glucose-6-phosphate dehydrogenase) are sensitive to oxidants and also defective in adaptation to oxidative stress (Kim et al. 2011). In cytosol, furthermore, the mitochondrial function requires maintaining redox homeostasis, which is mediated primarily by the voltage dependent anion channel (VDAC; porin pore). The VDAC releases superoxide anion from mitochondria to cytosol (Han et al. 2003). These results were observed in this study (Fig. 3b). Porin expression was apparently upregulated during H_2O_2-induced oxidative stress in the TG cells, as compared to the WT cells. Thus, expression of BrGR affects the cellular antioxidant capacity and the ability to withstand oxidative stress, which makes it easy to modify neutralizing ROS or to repair oxidative damage in the TG cell.

BrGR-expressing transgenic yeast cells activated molecular chaperones containing Hsp104, Hsp90, Hsp70, Hsp42, Hsp26, Cpr1p, Grp1, Sti and a zinc finger protein Zpr1 (Fig. 3a). The molecular chaperones that participate broadly in protein folding, Hsp104, Hsp90, Hsp70 family, and small Hsps (sHsps), promote the folding process through cycles of substrate binding and by cofactor proteins, which increase proteostasis under oxidative stress (Hartl and Hayer-Hartl 2002). Hsp104 and Hsp70 act together with Hsp26 in protein refolding after stress-induced unfolding. Also, Hsp90 depends on its association with a variety of cochaperones and cofactors. Cochaperones include Hsp70 and Hsp40 (Hsp42), and cyclophlins (Guo et al. 2007). The formation of a complex between Hsp70 and Hsp90 is mediated through the association of both chaperones to an adaptor protein termed an activator of the Ssa proteins Sti1 and an essential ER chaperone Grp (Grp94) that functions as an interaction domain of Hsp90 (Kim et al. 2011). In mammalian cells, an interaction between glutathione and sHsps resulted in increased resistance to cell death induced by hydrogen peroxide (Mehlen et al. 1996). In addition, Zpr1 is a zinc finger (ZnF) protein that translocates to the nucleus in response to growth stimuli, which is a lethal partner with Cpr1p for PPIase activity. Recently, TAP-tag based systematic analysis showed protein–protein interactions of all known 63 chaperones in *S. cerevisiae* (Gong et al. 2009). Also, Zpr1 is essential for cell viability in diverse eukaryotic organisms containing yeast and mice. Reduction of Zpr1 expression in mammalian cells by antisense or siRNA knockdown causes defects in transcription, prevents DNA synthesis, and results in an accumulation of cells in the G_1 and G_2 phases of the cell cycle (Kim et al. 2011). To effectively operate Hsps under oxidative conditions, ATP accumulation via activation of metabolic enzymes such as Hxt, Fba1, Pgk, G6PDH, GAPDH and Ald (Fig. 3a) must be accompanied because the refolding of denatured and partly denatured proteins by molecular chaperones is an ATP-dependent manner (Hartl and Hayer-Hartl 2002). Taken together, these results suggest that a co-activation of chaperone network of physiological and genetic interactions by BrGR over-expression could play a major role in buffering the proteins' oxidation or promoting protein (re)folding in the presence of ATP produced by glycolysis under oxidative stress.

Because *S. cerevisiae* has been used since ancient times in the production of goods for human consumption it has generally regarded as safe (GRAS) status, and *S. cerevisiae*-related processes are well accepted by the general public. The BrGR-expressing TG strain was more tolerant than the WT strain to high ethanol concentrations (Fig. 8), high osmotic pressure (Fig. 7c), and inhibitory factors to fermentation processes (Figs. 6, 7), which illustrates the ability of this yeast strain to adapt to different conditions. Laboratory-scale batch fermentation was performed on the basis of these results. The ability of TG cells to ferment and grow on YG medium with a high glucose concentrations (20%) was similar to that of the WT cells (Fig. 9), which was caused by down-regulation of GR activity in the case of high glucose concentrations (Fig. 9e). Fermentation ability did not differ between the WT strain and the *glr1* mutant strain encoding glutathione reductase (data not shown). These results imply that the GR protein is probably inactivated in the presence of high-glucose concentrations. Cells in which GR activity was inhibited by the alkylating agent carmustine (BCNU) were highly susceptible to ROS-induced oxidative stress due to agents such as paraquat, zinc, copper, iron, cadmium, ozone, ultraviolet light (UV), and peroxides. Loss of GR activity has been reported in the liver and kidney of fish exposed to arsenic and in an isolated rat hematocyte subjected to ethanol (Cardoso et al. 2008). In yeast, GR activity is reported to be inhibited by

trehalose and ethanol in a dose-dependent manner, with 70% inhibition in the presence of 1.5 M trehalose and 15% ethanol (Sebollela et al. 2004). Although various studies have been performed on GR inactivation (Cardoso et al. 2008), GR inactivation mediated by high glucose concentrations is poor. The results of the present study suggests that GR is inactivated in the presence of high glucose concentrations, but the mechanism underlying this inactivation remains to be elucidated.

In conclusion, the results of this study showed that BrGR expression in *S. cerevisiae* significantly increases in stress tolerance by improving redox homeostasis and proteostasis after activation of GSH recycling and cell rescue proteins by BrGR in the presence of H_2O_2, or that BrGR may act as a suppressor in response to various ROS-induced oxidative stresses. If further studies are performed to determine a mechanism for avoiding GR activity inhibition in the presence of high glucose concentrations or prevent GR inactivation via application of a useful gene, the results of these studies will provide an important clue for improving stress-tolerance in yeast during the fermentation process.

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