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The Gene Encoding γ-Glutamyl Transpeptidase II in the Fission Yeast Is Regulated by Oxidative and Metabolic Stress

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γ-Glutamyl transpeptidase (GGT, EC 2.3.2.2.) catalyzes the transfer of the γ-glutamyl moiety from γ-glutamyl-containing compounds, notably glutathione (GSH), to acceptor amino acids and peptides. A second gene (GGTII) encoding GGT was previously isolated and characterized from the fission yeast *Schizosaccharomyces pombe.* In the present work, the GGTII-*lacZ* fusion gene was constructed and used to study the transcriptional regulation of the *S. pombe* GGTII gene. The synthesis of β-galactosidase from the GGTII-*lacZ* fusion gene was significantly enhanced by NO-generating SNP and hydrogen peroxide in the wild-type yeast cells. The GGTII mRNA level was increased in the wild-type *S. pombe* cells treated with SNP. However, the induction by SNP was abolished in the Pap1-negative *S. pombe* cells, implying that the induction by SNP of GGTII is mediated by Pap1. Fermentable carbon sources, such as glucose (at low concentrations), lactose and sucrose, as a sole carbon source, enhanced the synthesis of β-galactosidase from the GGTII-*lacZ* fusion gene in wild-type KP1 cells but not in Pap1-negative cells. Glycerol, a non-fermentable carbon source, was also able to induce the synthesis of β-galactosidase from the fusion gene, but other non-fermentable carbon sources such as acetate and ethanol were not. Transcriptional induction of the GGTII gene by fermentable carbon sources was also confirmed by increased GGTII mRNA levels in the yeast cells grown with them. Nitrogen starvation was also able to induce the synthesis of β-galactosidase from the GGTII-*lacZ* fusion gene in a Pap1-dependent manner. On the basis of the results, it is concluded that the *S. pombe* GGTII gene is regulated by oxidative and metabolic stress.

Keywords: Fission yeast, Fermentable carbon source, γ-Glutamyl transpeptidase, LacZ fusion, Pap1, Transcriptional regulation, *S. pombe*

Abbreviations: GGT, γ-glutamyl transpeptidase; GSH, glutathione; ONPG, *o*-nitrophenyl-β-D-galactoside; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-polymerase chain reaction; *S. pombe*, *Schizosaccharomyces pombe*; SNP, sodium nitroprusside.

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Introduction

γ-Glutamyl transpeptidase (GGT; EC 2.3.2.2), a membrane-bound enzyme ubiquitously distributed in living organisms, catalyzes the degradation of extracellular glutathione (GSH, L-γ-glutamyl–L-cysteinylglycine) by cleavage of the γ-glutamyl bond, allowing the supply of extracellular cysteine for intracellular synthesis of GSH. GGT is a key enzyme implicated in the homeostasis of intracellular reduced GSH and hence in the regulation of the cellular redox state. GGT cleavage of GSH and the subsequent recapture of cysteine allow living cells to maintain low levels of cellular reactive oxygen species (ROS) and thereby avoid apoptosis induced by oxidative stress (Karp *et al.*, 2001).

There have been known other diverse cellular functions of GGTs. In the budding yeast *Saccharomyces cerevisiae*, GGT on the vacuolar membrane plays a role in the vacuolar transport and metabolism of the GSH stored in the vacuole serving as an alternative nitrogen source during nitrogen starvation (Mehdi and Penninckx, 1997; Mehdi *et al.*, 2001). In *Bacillus subtilis*, GGT acts as a powerful exo-γ-glutamyl hydrolase participating in capsular poly (γ-glutamic acid) degradation to supply stationary-phase cells with constituent glutamate (Kimura *et al.*, 2004), and is also important in utilizing GSH as the sole sulfur source (Minami *et al.*, 2004). GGT, reported as a virulence and colonizing factor of *Helicobacter pylori*, plays an important role on its *in vitro*

growth (Gong and Ho, 2004). *H. pylori* GGT upregulates the expression of COX-2 and EGF-related peptides in human gastric cells through phosphatidylinositol-3 kinase and p38 kinase (Busiello *et al.*, 2004). Meningococcal GGT is processed into two subunits in *Neisseria meningitidis*, at the conserved amino acid, threonine 427, and the majority of meningococcal GGT is associated with inner membrane facing to the cytoplasmic side (Takahashi and Watanabe, 2004). In the environment of cysteine shortage, like in rat cerebrospinal fluid, meningococcal GGT provides an advantage for meningococcal multiplication by supplying cysteine from environmental γ-glutamyl-cysteinyl peptides (Takahashi *et al.*, 2004a). Then, detection of GGT activity is used as an identification marker for *N. meningitidis* (Takahashi *et al.*, 2004b). Besides, it has been reported that GGT stimulates the expression of the receptor activator of NF-κB ligand mRNA and protein from bone marrow stromal cells independent of its enzymatic activity (Niida *et al.*, 2004).

The expression of the *S. cerevisiae* GGT gene is highly induced in cells growing on a poor nitrogen source, such as urea, through the GATA zinc-finger transcription factors Nil1 and Gln3, with Gzf3, another GAZA zinc-finger protein, acting as a negative regulator of nitrogen-source control in the expression of GGT (Springael and Penninckx, 2003). The GGT activity in *Candida albicans* is influenced by the carbon and nitrogen sources, and its increased GGT activity is responsible for the rapid decrease of the intracellular GSH in *C. albicans* during the yeast-to-mycelium conversion (Gunasekaran *et al.* 1995; Manavathu *et al.* 1996). 4-Hydroxynonenal, an electrophilic end product of lipid peroxidation, increases the expression of GGT in rat alveolar type II cells through activation of the ERK and p38 MAPK pathways (Zhang *et al.*, 2005).

A structural gene for γ-glutamyl transpeptidase, later named GGTI, was previously cloned from the fission yeast *Schizosaccharomyces pombe*, and its transcription was induced by NO-generating SNP and GSH-depleting L-buthionine-(S,R)-sulfoximine (BSO) in a Pap1-dependent way (Park *et al.*, 2004). It was also induced by non-fermentable carbon sources and nitrogen starvation in a Pap1-independent way but not by fermentable carbon sources (Kim *et al.*, 2005). The gene encoding a second γ-glutamyl transpeptidase, GGTII, was also characterized from *S. pombe*, and its mRNA level was elevated by the treatment with GSH-depleting diethylmaleate (Park *et al.*, 2005). In this continuing work, we demonstrate that the GGTII gene is regulated by oxidative and metabolic stress on a transcriptional level.

Methods and Materials

Chemicals Ampicillin, bovine serum albumin (BSA), *o*-nitrophenyl-β-D-galactopyranoside (ONPG), L-leucine, uracil, adenine, D-glucose, sucrose, lactose, glycerol, and Bradford reagent were purchased from Sigma Chemical Co. (St. Louis, USA). Restriction enzymes (*Hin*dIII and *Bam*HI), T4 DNA ligase, RNaseA, and *Ex Taq* polymerase were obtained from TaKaRa Shuzo Co. (Shiga, Japan). Yeast extract and agar were obtained from Amersham Life Science (Cleveland, USA). TRIZOL® Reagent and M-MuLV reverse transcriptase were from Invitrogen (Groningen, Netherlands) and Q-BIOgene (Montreal, Canada), respectively. PCR primers were from TaKaRa Shuzo Co. (Shiga, Japan). All other chemicals used were of highest grade commercially available.

Strains and growth conditions *E. coli* strain MV1184 was used for subcloning, and *S. pombe* KP1 (h^+ *leu1-32 ura4-294*) and TP108-3C (h^- *leu1-32 ura4D18 pap1::ura4*$^+$) were used for regulation study. The yeast cells were grown in minimal medium (Kim *et al.*, 2002), which contains KH phthalate (3 g), $Na_2HPO_4 \cdot 2H_2O$ (2.76 g), NH_4Cl (5 g), D-glucose (20 g), 1,000X vitamins stock (1 ml), 10,000X minerals stock (0.1 ml), 50X salts stock (20 ml), and L-leucine (250 mg) per 1 L. Vitamins stock (1,000X) contains 81.2 mM nicotinic acid, 55.5 mM inositol, 40.8 mM biotin, and 4.2 mM pantothenic acid. Minerals stock (10,000X) contains 8.1 mM H_3BO_3, 2.37 mM $MnSO_4$, 1.39 mM $ZnSO_4 \cdot 7H_2O$, 0.74 mM $FeCl_3 \cdot 6H_2O$, 0.25 mM $MoO_4 \cdot 2H_2O$, 0.6 mM KI, 0.16 mM $CuSO_4 \cdot 5H_2O$, and 4.76 mM citric acid. Salts stock (50X) contains 5.2 mM $MgCl_2 \cdot 6H_2O$, 0.1 mM $CaCl_2 \cdot 2H_2O$, 13.4 mM KCl, and 0.28 mM Na_2SO_4. The 2-day-grown culture was diluted 50-fold for inoculation. The yeast cells were shaken at 30°C, and the yeast growth was monitored by the absorbance at the wavelength of 600 nm.

Cell harvest and disruption The appropriate number of the yeast cells was harvested by centrifugation. The cellular pellets were resuspended in 20 mM Tris buffer (pH 7.4)-2 mM EDTA (buffer A) and disrupted using a glass bead beater and a sonicator. Supernatant was obtained after centrifugation and used as crude extract for both enzyme assay and protein determination.

Construction of the GGTII-*lacZ* fusion gene To separately monitor the expression of the *S. pombe* GGTII gene, the GGTII gene was fused into the promoterless β-galactosidase gene of the shuttle vector YEp367R as follows. The upstream sequence of the *S. pombe* GGTII gene was amplified from the original plasmid pPHJ02 (Park *et al.*, 2005) by PCR using synthetic primer 1 (5'-gct gcaggaattcaagcttagaaaggaatatatt-3') and primer 2 (5'-ggtagtgtctgta ggatccattacgaaacca-3') which contained *Hin*dIII and *Bam*HI sites, respectively. PCR was performed as described in the user's sheet offered by TaKaRa Shuzo Co. The PCR condition used was 94°C (denaturation, 1 min), 59°C (annealing, 1 min), and 72°C (extension, 1 min) for 30 cycles. The amplified DNA fragment was digested with *Hin*dIII and *Bam*HI, and ligated into the vector YEp367R digested with *Hin*dIII and *Bam*HI. After the transformation into the *E. coli* strain MV1184, the desired subclone was confirmed by restriction mapping and nucleotide sequencing, and named pGT04. The fusion plasmid pGT04 contains the 940 bp upstream sequence of the GGTII gene ranging –940 to +1 from the translational initiation site. The fusion plasmid pGT04 was used to monitor the expression of the GGTII gene without the interference of the other GGT genes in *S. pombe*.

β-Galactosidase assay β-Galactosidase activity in extracts was determined at 25°C by spectrophotometric method using ONPG as a substrate (Guarente, 1983). Its specific activity was represented in ΔA_{420}/min/mg protein.

Protein determination Protein content in extracts was determined according to the procedure of Bradford using BSA as a standard (Bradford, 1976).

RT-PCR analysis Total RNA was isolated from appropriate *S. pombe* cells using TRIZOL® reagent according to the manufacturer's protocol. First-strand cDNA was synthesized from 2 μg total RNA using M-MuLV reverse transcriptase (Lee *et al.*, 2004). One twentieth of the synthesized first-strand cDNA was used as templates in PCR using primer 3 (5'-aaattgctggaacccaatgttaaac-3') and primer 4 (5'-gttttactaagggagatgttattgcgaatt-3'). PCR was performed using *Ex Taq* polymerase as follows: denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. The PCR product was approximately 700 bp.

Results and Discussion

In the fission yeast *S. pombe* which resembles higher eukaryotes in aspects of genetics and physiology, there are 3 plausible GGT genes estimated from its nucleotide sequence stored in the GenBank database. To elucidate physiological roles and regulation of GGT in the fission yeast, the first *S. pombe* GGT gene, GGTI, was previously isolated and characterized (Park *et al.*, 2004). It is involved in the response against oxidative stress, and its transcription is enhanced by SNP and GSH depletion in a Pap1-dependent way (Park *et al.*, 2004). It is also induced by non-fermentable carbon sources, such as glycerol, acetate and ethanol, in a Pap1-independent way (Park *et al.*, 2004; Kim *et al.*, 2005). Its response against stress appears to be subject to both Pap1-dependent and Pap1-independent modes, which depends on stressful agents. The GGTII gene isolated from the same yeast cells is able to encode a protein of the same amino acid sequence with GGTI, although the nucleotides sequences in both coding regions are

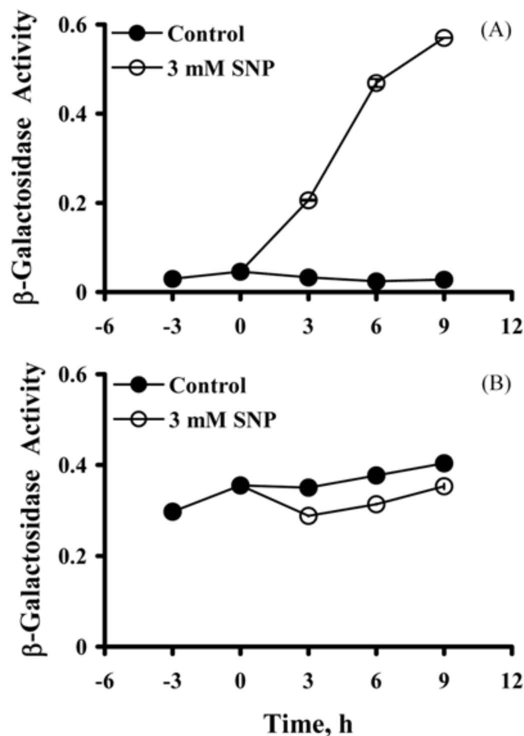

Fig. 1. Effect of sodium nitroprusside (SNP) on the synthesis of β-galactosidase from the fusion plasmid pGT04 in *S. pombe* wild-type (A) and Pap1-negative TP108-3C (B) cells. The yeast cells harboring plasmid pGT04 were grown in minimal medium, and split at the early exponential phase. Solid circles (-●-) indicate the untreated cells in β-galactosidase activity. SNP (-○-, 3 mM) was added into the appropriate culture flask. The β-galactosidase activity was determined at 25°C by the spectrophotometric assay using ONPG as a substrate, and its specific activity was expressed as ΔA_{420}/min/mg protein.

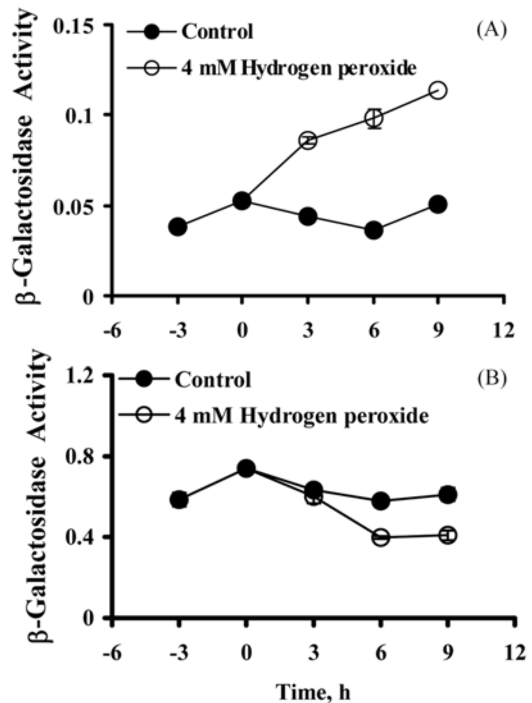

Fig. 2. Effect of hydrogen peroxide on the synthesis of β-galactosidase from the fusion plasmid pGT04 in *S. pombe* wild-type (A) and Pap1-negative TP108-3C (B) cells. The yeast cells harboring plasmid pGT04 were grown in minimal medium, and split at the early exponential phase. Solid circles (-●-) indicate the untreated cells in β-galactosidase activity. Hydrogen peroxide (-○-, 4 mM) was added into the appropriate culture flask. The β-galactosidase activity was determined at 25°C by the spectrophotometric assay using ONPG as a substrate, and its specific activity was expressed as ΔA_{420}/min/mg protein.

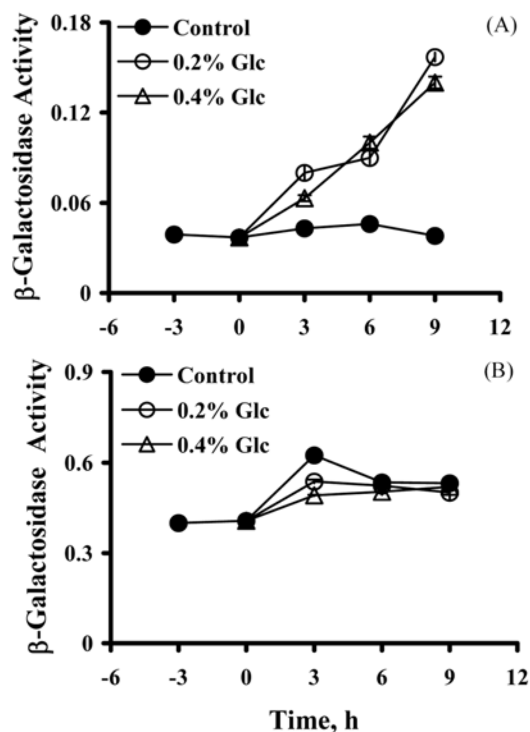

Fig. 3. Effect of glucose at low concentrations on the synthesis of β-galactosidase from the fusion plasmid pGT04 in *S. pombe* wild-type (A) and Pap1-negative TP108-3C (B) cells. The yeast cells harboring plasmid pGT04 were grown in minimal medium, and split at the early exponential phase. Solid circles (-●-) indicate the untreated cells in β-galactosidase activity. Glucose (-○-, 0.2%; -△-, 0.4%), as a sole carbon source instead of 2% glucose, was added into the appropriate culture flask. The β-galactosidase activity was determined at 25°C by the spectrophotometric assay using ONPG as a substrate, and its specific activity was expressed as ΔA_{420}/min/mg protein.

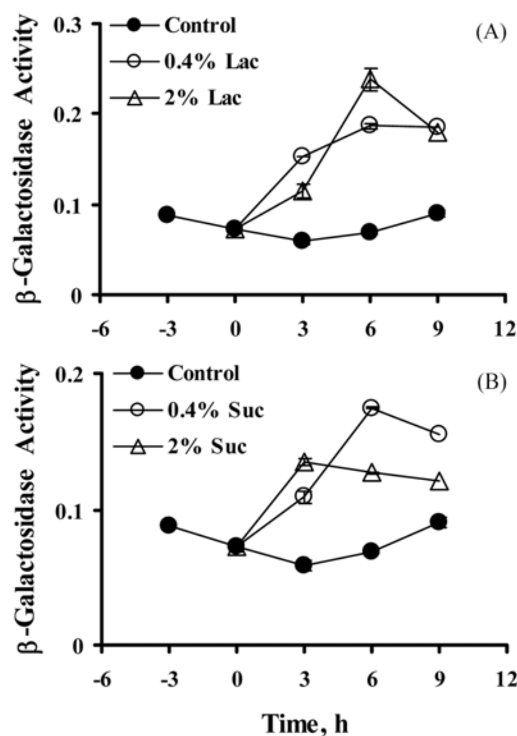

Fig. 4. Effects of fermentable carbon sources, such as lactose and sucrose, on the synthesis of β-galactosidase from the fusion plasmid pGT04 in wild-type *S. pombe* cells. The yeast cells harboring plasmid pGT04 were grown in minimal medium, and split at the early exponential phase. Solid circles (-●-) indicate the untreated cells in β-galactosidase activity. Lactose (Lac, A) or sucrose (Suc, B), as a sole carbon source instead of 2% glucose, was added into the appropriate culture flask (-○-, 0.4%; -△-, 2%). The β-galactosidase activity was determined at 25°C by the spectrophotometric assay using ONPG as a substrate, and its specific activity was expressed as ΔA_{420}/min/mg protein.

different (Park *et al.*, 2005). Accordingly, the two GGT genes have different upstream sequences, which might suggest susceptibility to differential regulation.

Nitrosative and oxidative stress Living cells are equipped with various kinds of defense systems due to their ability to generate highly toxic oxygen-derived free radical species. Simultaneously, metals and oxygen play beneficial roles in enzymatic reaction, metabolism, and signal transduction (Liu and Thiele, 1997). Reactive nitrogen species (RNS) play several roles in physiological and pathological processes, such as metabolism, immunity, inflammation, signal transduction, transcriptional regulation and apoptosis (Marshall *et al.*, 2000; Nathan and Shiloh, 2000). To understand regulation of the GGTII gene against nitrosative and oxidative stress, we measured the synthesis of β-galactosidase from the GGTII-*lacZ* fusion plasmid pGT04 in the *S. pombe* cells. The GGTII-*lacZ* fusion plasmid pGT04 was introduced into *S. pombe* cells. The *S. pombe* culture harboring the fusion plasmid pGT04 was split at the early exponential phase, and treated with 3 mM SNP or 4 mM hydrogen peroxide (Fig. 1, Fig. 2). Though the growth of the yeast cells was slightly delayed by the treatments, it was not arrested (data not shown). As shown in Fig. 1A, SNP markedly enhanced the synthesis of β-galactosidase from the GGTII-*lacZ* fusion gene. The yeast cells grown in the presence of 3 mM SNP contained 6.2-, 19.5- and 20.4-fold higher β-galactosidase activity at 3, 6 and 9 h after the treatment compared with those in control culture (Fig. 1A). The treatment with 4 mM hydrogen peroxide also gave rise to an enhancement in the synthesis of β-galactosidase from the GGTII-*lacZ* fusion gene (Fig. 2A). However, the induction by 3 mM SNP and 4 mM hydrogen peroxide was not observed in the Pap1-negative TP108-3C cells (Fig. 1B, Fig. 2B). The GGTI gene is also induced by SNP but not by hydrogen peroxide (Park *et al.*, 2004). Collectively, the *S. pombe* GGTII gene is induced by nitrosative and oxidative stress.

Regulation by carbon sources Glucose is a powerful signaling molecule which promotes major metabolic changes

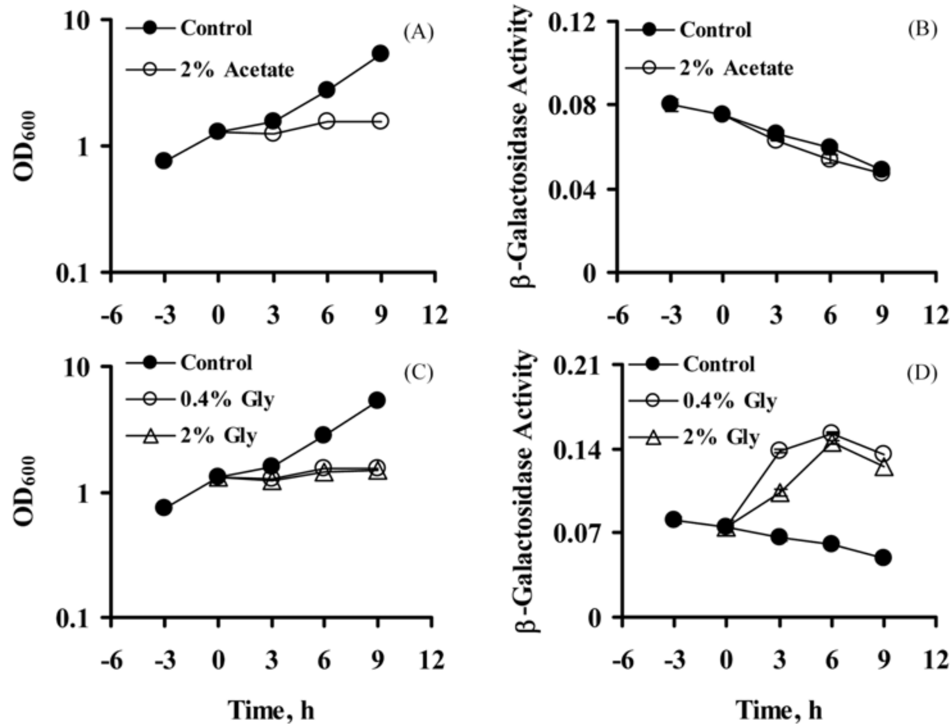

Fig. 5. Effects of non-fermentable carbon sources, such as potassium acetate and glycerol, on the synthesis of β-galactosidase from the fusion plasmid pGT04 in wild-type *S. pombe* cells. The yeast cells harboring plasmid pGT04 were grown in minimal medium, and split at the early exponential phase. Solid circles (-●-) indicate the untreated cells in growth curves (A, C) and β-galactosidase activity (B, D). Potassium acetate (-○-, 2%) or glycerol (-○-, 0.4%; -△-, 2%), as a sole carbon source instead of 2% glucose, was added into the appropriate culture flask. The β-galactosidase activity was determined at 25°C by the spectrophotometric assay using ONPG as a substrate, and its specific activity was expressed as ΔA_{420}/min/mg protein.

in living cells (Rolland *et al*., 2001). Metabolism of glucose produces compounds directly linked to the detoxification of intracellular hydroperoxides formed as byproducts by ongoing metabolic processes (Spitz *et al*., 2000). Therefore, deprivation of glucose causes a strong metabolic oxidative stress which is characterized by increased steady-state levels of intracellular hydroperoxides and glutathione disulfide (Lee *et al*., 1998; Song *et al*., 2002). Since the *S. pombe* GGTI gene is induced by non-fermentable carbon sources, effects of various carbon sources were examined on the synthesis of β-galactosidase from the GGTII-*lacZ* fusion gene. As a sole carbon source, glucose (low concentrations, 0.2%, 0.4%), lactose (0.4%, 2.0%), sucrose (0.4%, 2.0%), glycerol (0.4%, 2.0%) and acetate (2.0%) were substituted for 2% glucose in minimal medium (Fig. 3, Fig. 4, Fig. 5). As shown in Fig. 3, substitution of glucose at low concentrations markedly increased the synthesis of β-galactosidase from the GGTII-*lacZ* fusion gene. Glucose at lower concentration gave higher induction in the synthesis of β-galactosidase from the GGTII-*lacZ* fusion gene (Fig. 3). The glucose concentrations used did not change the growth of the yeast cultures largely (data not shown). Other fermentable carbon sources such as lactose and sucrose were also able to enhance the synthesis of β-galactosidase from the GGTII-*lacZ* fusion gene in the wild-type yeast cells, but each of them showed slightly different degrees of inductions (Fig. 4A, 4B). However, lactose nearly arrested the growth of the yeast cultures (data not shown). Growth of the budding yeast on non-fermentable carbon sources, such as glycerol, lactate, ethanol or acetate, requires the coordinated expressions of several genes, which are dispensable in the presence of substrates easily utilized by glycolysis (e.g. glucose or fructose) (Hiesinger *et al*., 2001). In an *S. pombe* culture containing the fusion plasmid pGT04 at the early exponential phase grown in supplemented minimal medium, a switch was made from 2% glucose to 2% potassium acetate, 0.4% and 2.0% glycerol or 2% ethanol as a sole carbon source. The β-galactosidase activity was measured at 3, 6 and 9 h after the switch. A switch to potassium acetate and glycerol gave rise to an arrest in the growth of the treated culture (Fig. 5A, Fig. 5C). A switch to glycerol could induce the synthesis of β-galactosidase from the fusion gene in the wild-type *S. pombe* cells (Fig. 5D). However, a switch to 2% potassium acetate could not affect the synthesis of β-galactosidase from the GGTII-*lacZ* fusion gene (Fig. 5B). A switch to 2% ethanol, the other non-fermentable carbon source used in this study, showed similar patterns in growth and β-galactosidase of the yeast cultures (data not shown). It was previously shown that the GGTI gene was induced by non-fermentable carbon sources but not by fermentable carbon sources (Kim *et al.*, 2005). From the

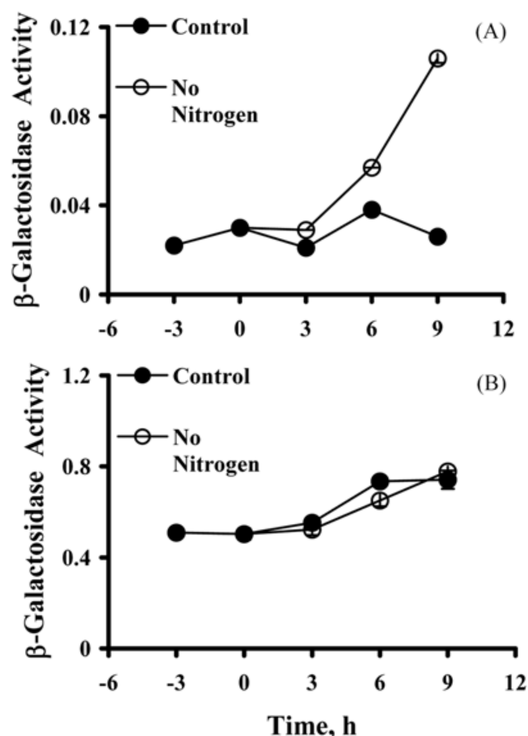

Fig. 6. Effect of nitrogen starvation on the synthesis of β-galactosidase from the fusion plasmid pGT04 in *S. pombe* wild-type (A) and Pap1-negative TP108-3C (B) cells. The yeast cells harboring plasmid pGT04 were grown in minimal medium, and split at the early exponential phase. Solid circles (-●-) indicate the untreated cells in β-galactosidase activity. Ammonium chloride, a nitrogen source in the untreated cells, was starved in the appropriate flask (-○-). The β-galactosidase activity was determined at 25°C by the spectrophotometric assay using ONPG as a substrate, and its specific activity was expressed as ΔA_{420}/min/mg protein.

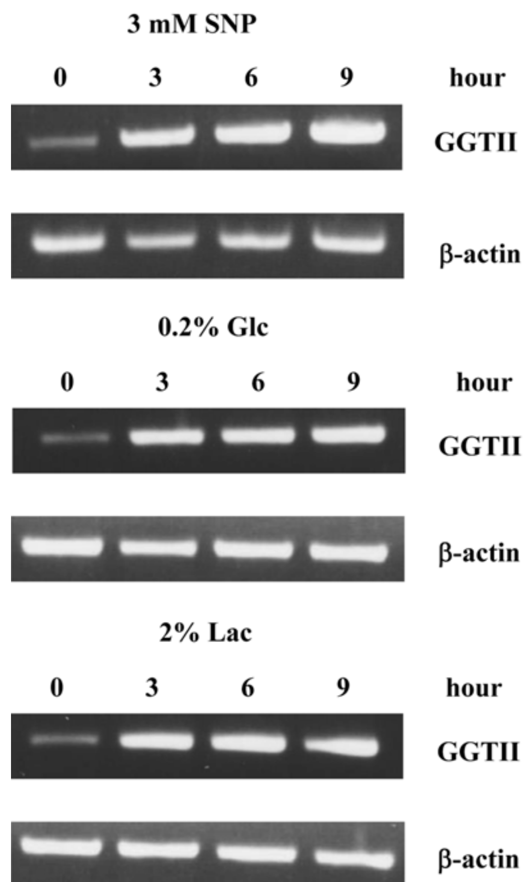

Fig. 7. Variations in the GGTII mRNA level of the wild-type *S. pombe* cells treated with 3 mM sodium nitroprusside (SNP), 0.2% glucose (Glc) or 2% lactose (Lac). Total mRNA was isolated at 3, 6 and 9 hours after the treatments. The GGTII mRNA was detected using RT-PCR as described in 'Materials and Methods'. A representative of three separate experiments is shown.

current and previous studies, glycerol was found to induce both GGTI and GGTII genes in *S. pombe*, the physiological meaning of which still remains to be solved. Taken together, the results indicate that the two GGT genes are subject to differential regulation responding carbon sources.

Nitrogen starvation Adaptation of cells to nutritional stress is paralleled by alterations in the expression of the responsible genes, which may be associated with selective degradation of superfluous proteins. In *S. cerevisiae*, expression of the *CIS2* gene encoding GGT, the main GSH-degrading enzyme, is highest in cells growing on a poor nitrogen source, such as urea (Springael and Penninckx, 2003). During growth on a preferred nitrogen source, like NH_4^+, *CIS2* expression is repressed through the mechanism involving the Gln3-binding protein, Ure2/GdhCR (Springael and Penninckx, 2003). When ammonium-grown *S. pombe* KP1 cells harboring the fusion plasmid pGT04 were transferred to a minimal medium lacking a nitrogen source, the synthesis of β-galactosidase from the GGTII-*lacZ* fusion gene was significantly enhanced (Fig. 6). Six hours after the exposure to nitrogen-starved conditions, the synthesis of β-galactosidase in the yeast cells rose 4.1-fold (Fig. 6). In conclusion, the expression of the *S. pombe* GGTII gene, like the GGTI gene (Kim *et al.*, 2005), is induced under nitrogen starvation.

Pap1-dependence The yeast cells are known to induce the transcription of genes required for detoxification of stressful agents (Lee *et al*., 2003). The two transcription factors, Pap1 and Atf1, are believed to be responsible for the induction of stress-related genes in the fission yeast *S. pombe* (Nguyen *et al*., 2000). To find whether Pap1 is involved in the induction of the *S. pombe* GGTII gene by SNP, fermentable carbon sources and nitrogen starvation, the Pap1-negative *S. pombe* strain TP108-3C was used. The TP108-3C cells harboring the fusion plasmid pGT04 were exposed to SNP, fermentable carbon sources and nitrogen starvation, and the β-galactosidase activities in the treated cells were measured. The TP108-3C cells harboring the fusion plasmid could not gave rise to any changes in the synthesis of β-galactosidase from the GGTII-

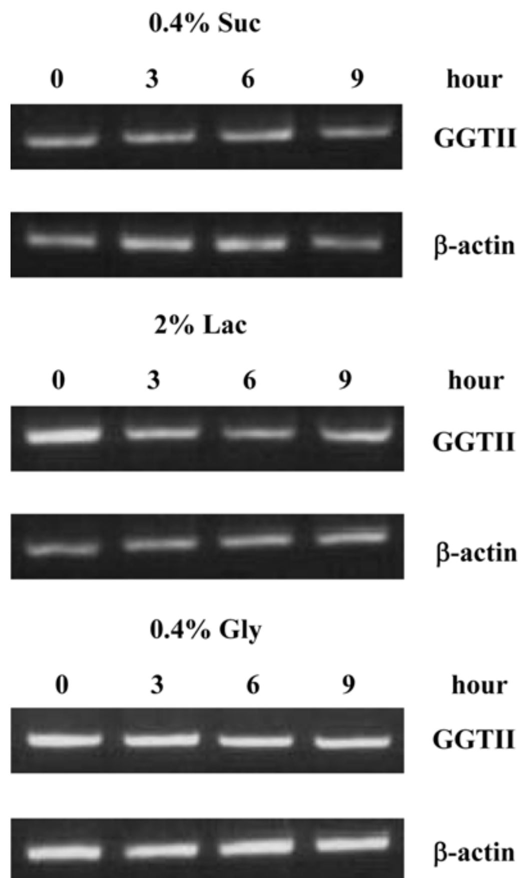

Fig. 8. Variations in the GGTII mRNA level of the Pap1-negative mutant TP108-3C cells treated with lactose, sucrose or glycerol as a sole carbon source. Total mRNA was isolated at 3, 6 and 9 hours after the treatments. The GGTII mRNA was detected using RT-PCR as described in 'Materials and Methods'.

lacZ fusion gene when treated with SNP (Fig. 1B), glucose at low concentrations (Fig. 3B) and nitrogen starvation (Fig. 6B). These results indicate that the GGTII gene is regulated by SNP, carbon sources and nitrogen starvation in a Pap1-dependent manner. Though the induction by SNP of the GGTI gene is also mediated by Pap1, its induction by nitrogen starvation does not require the presence of Pap1 (Park *et al.*, 2004; Kim *et al.*, 2005). Therefore, it is assumed that the two GGT genes are subject to differential mechanisms to induce their expressions in response to nitrogen starvation.

mRNA level To confirm the induction of the GGTII gene on the transcriptional level, the GGTII mRNA level was measured using RT-PCR in the wild-type *S. pombe* cells treated with 3 mM SNP, 0.2% glucose, and 2% lactose (Fig. 7). As shown in Fig. 7, the GGTII mRNA level was significantly increased after all the treatments. However, the GGTII mRNA level in the Pap1-negative TP108-3C cells was not increased by sucrose, lactose and glycerol (Fig. 8). These results confirm that the transcriptional induction of the GGTII gene occurs in a Pap1-dependent manner. In brief, the GGTII gene is transcriptionally regulated by nitrosative stress and fermentable carbon sources. In shift experiments, it was observed that the β-galactosidase activity in TP108-3C cells was much higher than that in wild-type cells. To explain this fact, the GGTII mRNA level was detected in wild-type and TP108-3C cells at early and mid- exponential phases (Fig. 9). The TP108-3C cells gave rise to much higher GGTII mRNA level at early exponential phase than the wild-type cells, which corresponded with the results obtained with the GGTII-*lacZ* fusion gene. This suggests that the basal expression of the GGTII gene may be repressed by Pap1, at least in early exponential phase.

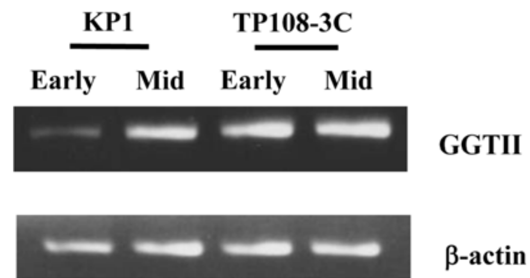

Fig. 9. Expression patterns of the GGTII gene in wild-type KP1 and Pap1-negative TP108-3C *S. pombe* cells. The cells were harvested at the early and mid- exponential phases, which were denoted as 'Early' and 'Mid', respectively. RT-PCR analysis was preformed to detect gene expression using specific primers for GGTII and β-actin mRNAs.

The yeast growth in the absence of glucose relies on the breakdown of partially oxidized substrates that are derived from fermentative reactions. The carbon source-responsive element (CSRE) mediates transcriptional activation of the gluconeogenic genes during the growth of the yeast *S. cerevisiae* on non-fermentable carbon sources (Vincent and Carlson, 1998). A few plausible CSRE motifs, which corresponds to the consensus CCN_6CCG sequence typical of a subfamily among zinc cluster proteins (Walther and Schuller, 2001), are identified in the upstream regions of both GGTI and GGTII genes (Fig. 10). Though their roles remain unknown, one or some CSRE motifs might work for the induction of the GGTI gene by non-fermentable carbon sources.

There are two known stress signal pathways in the fission yeast *S. pombe*. The one is the Spc1-Atf1 pathway in which stress-activated Spc1 induces Atf1, and the other is mediated by Pap1 independently of Spc1 activation (Nguyen *et al.*, 2000). The expression of the *S. pombe* glutathione synthetase gene was reported to be regulated by the Spc1-Atf1 pathway (Kim *et al.*, 2003). A transcription factor Pap1 required for survival to oxidative stress shows high homology to mammalian c-Jun like its *S. cerevisiae* homologue Yap1 (Toda *et al.*, 1991). The *S. pombe* cells deleted in *pap1*$^+$ show high sensitivity to oxidative stress but not to osmotic stress or nutrient deprivation (Toone *et al.*, 1998). Spc1 is activated by high osmolarity, oxidative stress, and heat shock (Millar *et al.*, 1995; Shiozaki and Russell, 1995; Kato *et al.*, 1996). Several

attgggcattgaaatactatttgtccttgataaaatctttgcccatgtaaataaatccaggattagact GGTI
aaagtgagaaaggaatatattgaagtaagttaccgctactcaaatgagatgaatgctaaaagaatgcgtt GGTII

aacacatcaaaatttgaattaatcgatgagttaaaatgaatcgcttcgaccctattatattagttttat GGTI
actgcgtcttcactccagagaatatggctctgagtagcttacgaatctgcgttaaaaagctgtgactat GGTII

atcaactataagaaaaagttcctaaaaacaatttgtaatatcataaacatcgatgttgcaaaccattat GGTI
gaaatcagagtaaatggcatgttgcatgaggatcaggcggaatcgacagtttacatggtcatccaaaatc GGTII

tagacagatagatgaaagactacaaaatcaaatacatttaatacggatttctattatctatctttcatg GGTI
aatccgttacacaacaactttcacattgctgtcggaaagaaagtcttgttatagttaacaggcaatt GGTII

gaatatccattttcaaagtgctcattcataaacattgaatgaattgtgcattcatcgccatattattcgg GGTI
tacatgataaagctagtatagacaaaactctgtaaacgacaatctacgattaaactcaacaaagctacg GGTII

cctcgcttactagtcgatcaattgggattttcgaaggatctacctatcgatttagaaatttttgccaat GGTI
aaatgtttcccatcaacgtgtgacttagtttctgatactttgtttagtttaaacatactggaaacatgac GGTII

atgcggttttaaaaacaaaagatatgaaagatatttagtcagtaaaatgcatttgttagttttgccgg GGTI
atttacatttgtttatccatcacaggccatcgctaggacgccctttacgcacaacattcgtaactgtag GGTII

cttcacagtcaaaacatgttttcggttgtataaattagatatgtggtttgctccaccaatttgcctccc GGTI
acagtgctgtatgatttaacgagagaacgcttatttcactacttgtgatacgaaagagagtagcattt GGTII

gcttatttctctcaacttgcacgcacacagcaaactcgagccgttctcttgagaagcaaggatttcttcc GGTI
ataaccttgtgataaaggctgaagcaggatgagtgaaatgataaagtgacacgttatcgatcattaaaca GGTII

gttgaatttatttaaagctaacgctattcaattattgattagccttgttgatttcttcatgctaatatcg GGTI
acacctgcgcttagataatttctcaacacttgagttattcaactgttctcgttacccttgaaaacagtt GGTII

taaaaaaaggtgtagaagttgcggttggctttctttagtccatttgggtcattttccccaaaaatta GGTI
ggtttctagagaattaagaggatttctaaattattgatcaaatttatctgttgtttctctcagttgct GGTII

aatttgttaaattagaagttcttttcatttttcttcatccataccctcttctcaaaggtttgtgcaagat GGTI
ttcgttgattataattgtcgctcgttgagtttaatcggtataaaagcgagaacattagaagtttggcg GGTII

tccttaatcttggtaaaggtatttttttaaattaatttcattccatttcatacttgtttttttttt GGTI
tctgaaagaagctagctcctttttttactttcattttttttctttaatctgtgtgtaaatagctttaagt GGTII

ttggatatttcagcactttttaaattgtg**atg** GGTI
cattgtgtaataattttttttggtttcgta**atg** GGTII

Fig. 10. Comparison in the upstream sequences of the two GGT genes, GGTI and GGTII, in the fission yeast *S. pombe*. Plausible Pap1 binding sites are underlined, whereas plausible CSRE motifs are boxed.

reports have shown that carbon starvation is an environmental stress able to activate the MAPK Sty1 in *S. pombe* (Stelttler *et al*., 1996; Shiozaki *et al*., 1997). Recently, glucose limitation in *S. pombe* not only promotes activation of the SAPK signaling pathway resulting in increased expression of Atf1-dependent stress-related genes but also induces an oxidative stress that favors the concerted expression of additional genes depending on the transcription factor Pap1 (Madrid *et al*., 2004). The consensus sequence for the binding site of the Pap1 protein was previously determined to be TTACGTAA (Fujii *et al*., 2000). The five plausible Pap1 binding sites are found at the upstream region of the *S. pombe* GGTII gene, whereas the only plausible Pap1 site is located at the upstream region of the GGTI gene (Fig. 10). Some of them may be responsible for the regulation by the Pap1 protein in the induction of the *S. pombe* GGTI and GGTII genes. Atf1 is most homologous to ATF-2, a key substrate of the human SAPKs, p38 and c-Jun N-terminal kinases (Gupta *et al.*, 1995; Livingstone *et al.*, 1995; van Dam *et al.*, 1995; Raingeaud *et al.*, 1996), which underscores the high conservation between the *S. pombe* and human SAPK pathways. The consensus sequence for the binding site of the Atf1 protein was previously determined to be TGAGGTCAG (Degols and Russell, 1997). Plausible Atf1 binding sites are not identified at the upstream regions of the *S. pombe* GGT genes (Fig. 10). However, the detailed mechanisms in the regulation of the *S. pombe* GGT genes requires further approaches.

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