Effects of glucose sensing/signaling on oxidative stress response in glucose repression mutants of *Schizosaccharomyces pombe*

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ABSTRACT. The resistant to glucose repression mutants of *Schizosaccharomyces pombe* (*ird5*, *ird13*, and *ird14*) have a high tolerance to oxidative stress induced by H_2O_2. In all *ird* mutants, the increased expression level of the *fbp1* gene can be interpreted as a lack of glucose repression in these mutants. To investigate the mechanisms of the oxidative stress response in *ird* mutants, we analyzed the transcription of stress response-related genes, *sod1*, *ctt1*, *atf1*, *pap1*, and *sty1*, under stressed and non-stressed conditions. We then analyzed the phosphorylation state of the Sty1-MAP kinase in *ird* mutants. Our findings support the concept of an adaptive response to oxidative stress in these mutants. In addition, these results imply that either glucose signaling mechanisms leading to glucose repression and glucose utilization as an energy source are regulated apart from each other or, like *Saccharomyces cerevisiae*, *S. pombe* might have additional glucose detection systems.

Key words: *Schizosaccharomyces pombe*; Glucose detection; Oxidative stress response; Glucose repression; *fbp1*; *hxk2*

INTRODUCTION

Glucose plays important regulatory roles in the expression of many genes related to glucose sensing/signaling, glucose transport, glucose catabolism, utilization of alternative carbon sources, and stress response pathways (Carlson, 1999; Johnston, 1999; Rolland et al., 2002; Chen et al., 2003, 2008). Unlike *Saccharomyces cerevisiae*, regulatory mechanisms in glucose sensing and signaling are not fully understood in fission yeast (*Schizosaccharomyces pombe*). Nevertheless, it has been reported that glucose is detected by the Git3 G protein-coupled receptor, which generates an intracellular signal via the cyclic AMP (cAMP)-dependent protein kinase A (PKA) pathway in *S. pombe* (Welton and Hoffman, 2000; Hoffman, 2005). Multiple genes (*ght1-ght6*) encoding hexose transporters have also been identified in *S. pombe* (Heiland et al., 2000). Recently, an Ssp2 Snf1-like protein kinase, which regulates glucose repression/de-repression, has been identified in *S. pombe* by Matsuzawa et al. (2012).

Glucose sensing also triggers the activation of adenylate cyclase to elevate cAMP, which results in PKA repression of the transcription of the *fbp1* gene that encodes fructose-1,6-biphosphatase in *S. pombe* (Hoffman and Winston, 1991; Byrne and Hoffman, 1993). PKA inhibits conjugation, sporulation, respiration, thermotolerance, and utilization of alternative carbon sources (DeVoti et al., 1991; Maeda et al., 1990, 1994; Fernandez et al., 1997; Riberio et al., 1997).

Glucose starvation potentiates a stress-activated mitogen-activated protein kinase (MAPK) pathway. MAPK cascades are well-known, highly conserved signal transduction pathways possessing three protein kinases, the MAPK, the MAPK kinase (MAPKK), and the MAPKK kinase (MAPKKK) (Degols et al., 1996; Stettler et al., 1996; Samejima et al., 1997; Shiozaki et al., 1997). One of the components is the spc1/sty1 MAPK, which plays an important role in the regulation of downstream targets by triggering two transcriptional activators, Atf1-Pcr1 and Pap1 (Ikner and Shiozaki, 2005). Sty1 is required for many processes that are also negatively regulated by PKA, including mating and sporulation, gluconate transport, and thermotolerance (Shiozaki and Russell, 1996; Wilkinson et al., 1996). Neely and Hoffman (2000) showed that this pathway regulated the transcription of the *fbp1* gene by protein-DNA interactions at upstream activation site 1 (UAS1) and UAS2 of this gene.

On the other hand, the high resistance to oxidative stress under glucose depletion either involves the induction of a wide array of stress-responsive genes due to carbon stress (Madrid et al., 2004) or is a defense mechanism against the increased accumulation of reactive oxygen species that are derived from aerobic respiration (Roux et al., 2009).

Lin et al. (2002) reported that an *S. cerevisiae* strain with a deletion of the *HXK2* gene, which is involved in glycolysis, mimics the effect of growth in low glucose and extends the life span. Recently, lowering caloric intake by limiting glucose was found to extend life span in fission yeast (Chen and Runge, 2009; Roux et al., 2010; Zuin et al., 2010).

In this study, we investigated the effects of glucose sensing/signaling on stress-activated MAPK pathways using glucose repression-resistant mutant strains (*ird5*, *ird13*, and *ird14*) of *S. pombe*.

MATERIAL AND METHODS

Chemicals and reagents

Anti-phospho-p38 MAPK (Tyr182) polyclonal antibody and goat anti-rabbit IgG

(horseradish peroxidase-conjugated) antibody were purchased from Thermo Scientific, USA. ECL-Plus Western Blotting Detection system was purchased from Amersham, USA. Other reagents were obtained from Sigma Aldrich, USA.

Yeast strains and growth media

S. pombe Lindner *liquefaciens* (wild type, 972h^-) and glucose repression resistant constitutive invertase mutants (*ird5*, *ird13*, and *ird14*) (Kig et al., 2005) were used in this study. The growth medium, containing 0.5% yeast extract, 3% sucrose, and 400 μg/mL 2-deoxy-D-glucose (Rincon et al., 2001) was used for the selection of *ird* mutants. The cells were grown in the standard rich yeast extract liquid or agar medium (YEL or YEA, respectively). The YEL medium contained 3% glucose (repressed condition) or 0.5% glucose (glucose starvation condition), and the YEA medium contained 0.1% glucose plus 3% glycerol (de-repressed condition).

Induction of oxidative stress

Exponentially growing *S. pombe* cells (wild type, *ird5*, *ird13*, and *ird14*) were split into two tubes. In the experimental group, 2 mM hydrogen peroxide (H_2O_2) (Sigma, H1009) was added to the medium. The control group was not exposed to oxidative stress. After 1 h, both experimental and control cells were removed by centrifugation, washed with sterile distilled water, and plated on YEA. After incubation at 30°C for 3-4 days, the survival rates were calculated as a percentage of the numbers of colonies obtained from the serial dilutions of control and experimental cells.

Hydrogen peroxide consumption by cells

At the end of oxidative stress implementation, the remaining H_2O_2 concentration in the medium was detected by the phenol-red method (Pick and Keisari, 1980) to check the H_2O_2 consumption of the cells. One milliliter of phenol-red buffer, which contained 40 mM NaCl, 10 mM potassium phosphate buffer, pH 7.0, 0.1 g/mL phenol-red, and 8.5 U/mL horseradish peroxidase, was added to a 500-μL cell suspension, and the reaction mixture was incubated at 37°C for 5 min. The absorbance of the resulting purple complex was measured at 550 nm. The concentration of H_2O_2 in the medium was calculated according to a standard graph prepared by using absorbance values of the mixtures containing 1, 10, 100, and 200 μM H_2O_2.

Cell disruption and extraction of soluble proteins

The yeast cells were homogenized with lysis buffer containing 50 mM Tris-HCl, pH 6.8, 5 mM ethylenediaminetetraacetic acid, 150 mM NaCl, 100 mM dithiothreitol, 10% glycerol, and protease inhibitor cocktail. The extract was centrifuged at 12,000 *g* for 20 min at 4ºC to remove insoluble material. The protein concentration of the supernatant was determined by the Bradford assay.

Polyacrylamide gel electrophoresis (PAGE) and Western blot analysis

For sodium dodecyl sulfate (SDS)-PAGE and Western blotting, protein extracts were

denatured in sample buffer and boiled for 3 min. Equal amounts of protein (50 μg/well) were analyzed by 10% SDS-PAGE. After electrophoresis, proteins were transferred to polyvinylidene fluoride membranes using a BIO-RAD Semi-Dry apparatus. Membranes were blocked with 5% bovine serum albumin fraction V (in Tris-buffered saline with Tween 20) at room temperature for 1 h. Primary antibody against the phospho-p38 MAPK was used at 1:500 for 1 h at room temperature. Peroxidase-conjugated goat anti-rabbit secondary antibody was used at 1:100 for 1 h at room temperature. Protein bands were detected with ECL-plus and quantitative analysis was performed with ChemiDoc MP and the ImageLab 4.0.1 Software (BIO-RAD).

RNA isolation and cDNA synthesis

Total RNA was isolated using a High Pure RNA Isolation Kit (Roche) following manufacturer instructions. First-strand oligo (dT)-primed cDNA synthesis was performed using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche) according to the manufacturer protocol.

Real-time polymerase chain reaction (PCR)

Quantitative real-time PCR (RT-PCR) was performed with the FastStart SYBR Green Master Kit (Roche), using the synthetic first-strand cDNA as a template, following manufacturer instructions. Briefly, the reaction mixture consisted of 25 μL FastStart SYBR Green Master, 0.2 μM forward and reverse primers, and 0.1 μg cDNA made up to 50 μL with ultrapure nuclease-free water. The PCR conditions were set as follows: 95°C for 10 min (preincubation), followed by 40 cycles of 95°C for 10 s, 53°C for 10 s, and 72°C for 20 s. The final step included a gradual temperature increase from 55° to 95°C at the rate of 1°C/10 s to enable melting curve data collection. A non-template control was run, and serial dilutions (1, 1:10, and 1:100) of the reference (actin gene, *act1*) and the target genes were included in every assay. The amplification specificity of each reaction was verified by melting curve analysis. Expression levels were normalized against the reference gene, *act1*. The relative gene expression levels were determined according to the method of Pfaffl (2001).

Statistical analysis

Data are reported as means ± standard deviation for three experiments. Statistical comparisons were made using one-way analysis of variance (ANOVA) for RT-PCR and Western blot analysis of GraphPad Prism 5. Differences in mean values were considered to be significant when $P < 0.05$.

RESULTS AND DISCUSSION

Resistance of *ird* mutants against oxidative stress

The survival rates of *ird* mutants and wild-type cells growing in different glucose concentrations (repressed, starvation, and de-repressed conditions) were compared under a mild oxidative stress. The *ird* mutants were found to be more resistant to oxidative stress than the

wild-type strain in each condition. All *ird* mutants and the wild-type strain exhibited resistance to oxidative stress when they were grown in glucose starvation or de-repressed conditions (Figure 1). These results supported our previous results related to *ird11* studies (Suslu et al., 2011; Palabiyik et al., 2012). These findings imply an adaptive response to oxidative stress in these mutants and links between glucose sensing/signaling and the oxidative stress response.
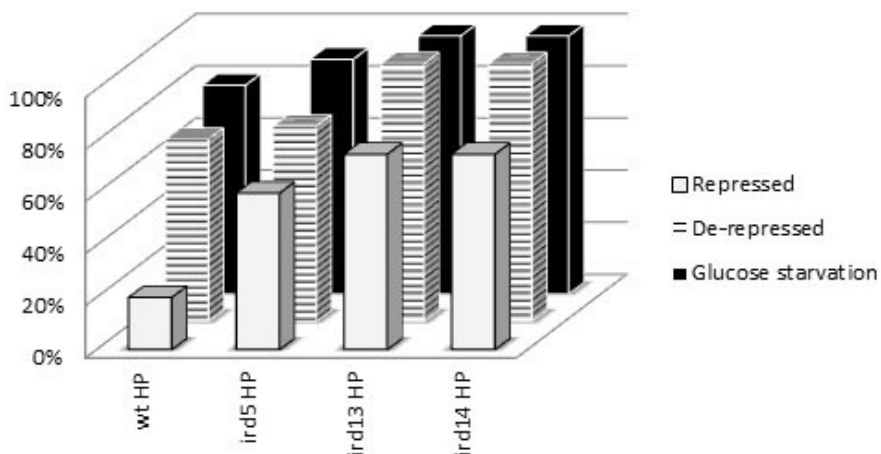

Figure 1. Survival rates of the wild-type and *ird* mutants under mild-oxidative stress condition. Exponentially grown mutant and wild-type cells in different glucose-containing media (0.1% glucose and 3% glycerol, 0.5% glucose and 3% glucose providing de-repressed, starvation and repressed conditions, respectively) were exposed to H_2O_2 for 1 h and were compared with untreated cells. wt = wild type; HP = treated with H_2O_2.

In addition, in this study, we checked that the H_2O_2 in the medium was entirely consumed by either the wild-type strain or *ird* mutants after 1 h of H_2O_2 treatment (data not shown). This finding indicated that the oxidative stress resistance of *ird* mutants did not arise from inadequate H_2O_2 uptake.

Ability to escape glucose repression in *ird* mutants

The expression profile of the *fbp1* gene in *ird* mutants was compared with that in the wild-type strain. The *fbp1* gene is used as a marker for glucose repression because it is transcriptionally repressed by glucose (Hoffmann and Winston, 1989, 1990), and it is induced by glucose starvation (Kanoh et al., 1996; Neely and Hoffman, 2000).

Under repressed conditions, *fbp1* expression in *ird5*, *ird13*, and *ird14* mutants was increased 4- to 8-fold compared to the wild-type strain (Figure 2). This finding is consistent with that of another study, which found reduced glucose consumption by *ird* mutants (Kig et al., 2005). Therefore, the elevated expression of the *fbp1* gene in *ird* mutants (Figure 2) might be because of a lack of glucose repression. In addition, the high resistance of *ird* mutants to oxidative stress might result from a lack of glucose repression because it is known that glucose depletion causes resistance to oxidative stress in *S. pombe* (Madrid et al., 2004; Roux et al., 2009).
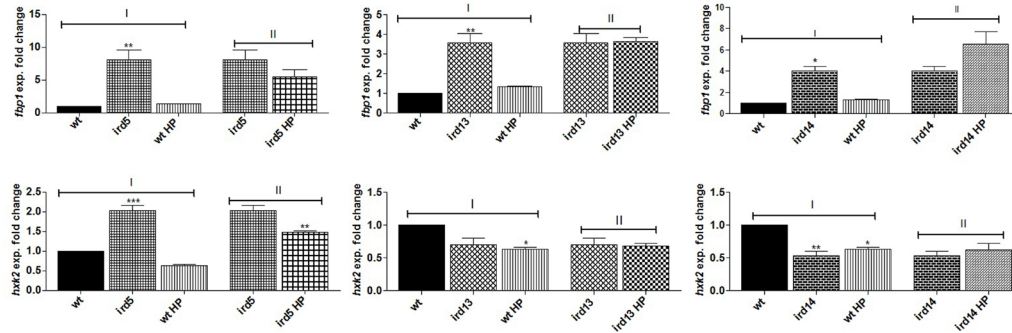
Figure 2. Expression levels of *fbp1* and *hxk2* genes. The expression levels of *fbp1* and *hxk2* genes in wild-type and *ird* mutant cells treated with H_2O_2 or untreated, after grown in rich media containing 3% glucose (repressed media), were determined according to Pfaffl method relative to the actin gene. Significant differences between **I.** untreated wild-type and untreated *ird* mutants and treated wild-type cells and **II.** untreated and treated *ird* mutants were evaluated by the Tukey test. ***$P < 0.0001$; **$P < 0.001$; *$P < 0.05$. wt = wild type; HP = treated with H_2O_2.

The *hxk2* gene, which is required for glucose metabolism (Petit et al., 1996), was analyzed in *ird* mutants and compared with the wild-type strain. The expression level of the *hxk2* gene increased approximately 2-fold in the *ird5* mutant (Figure 2) while it decreased in the *ird13* and *ird14* mutants compared to the wild-type strain. These results indicate that glucose can be metabolized faster in the *ird5* mutant than in the *ird13* and *ird14* mutants, as with the *ird11* mutant (Palabiyik et al., 2012). We suggest that the oxidative stress resistance of *S. pombe* may be associated with the glucose sensing/signaling pathways rather than the availability and metabolism of glucose.

## Oxidative stress resistance of *ird* mutants

To investigate the possible mechanism underlying the high oxidative stress resistance in *ird5*, *ird13*, and *ird14* mutants, the expression levels of the *ctt1* and *sod1* genes were examined. The expression levels of the *sod1* and *ctt1* genes were not increased in *ird* mutants under non-stressed conditions (Figure 3). With the exception of the *ird5* mutant, the expression level of the *sod1* gene increased in all mutant cell lines under stressed conditions. In addition, the expression level of the *ctt1* gene was significantly increased in the *ird14* mutant but not in the other mutants (Figure 3). These results suggest that the resistance of *ird* mutants to oxidative stress appears not to be related to the constitutive activation of either *sod1* or *ctt1*.

Atf1 and Pap1 are known to be involved in the regulation of the cellular defense mechanism(s) against increasing oxidative stress in aerobically growing, glucose-depleted cells (Madrid et al., 2004). These transcription factors have been proposed to be activated under mild oxidative stress conditions in *S. pombe* (Quinn et al., 2002). In this study, a mild oxidative stress condition was induced to specifically activate the Atf1 and Pap1 transcription factors. As expected, the expression levels of *atf1* and *pap1* were increased in wild-type cells under mild oxidative stress, as well as in all three mutant cell lines. With the exception of *idr5*, the expression level of *atf1* did not significantly increase in the mutants under non-stressed conditions. However, *pap1* expression levels were significantly higher in the *ird5*, *ird13*, and *ird14* mutants than in the wild-type strain under stressed conditions (Figure 4). These results

suggest that the resistance of the *ird5* and *ird13* mutants to oxidative stress appears to be related to the constitutive activation of *pap1*, but the resistance of the *ird14* mutant to oxidative stress is not.
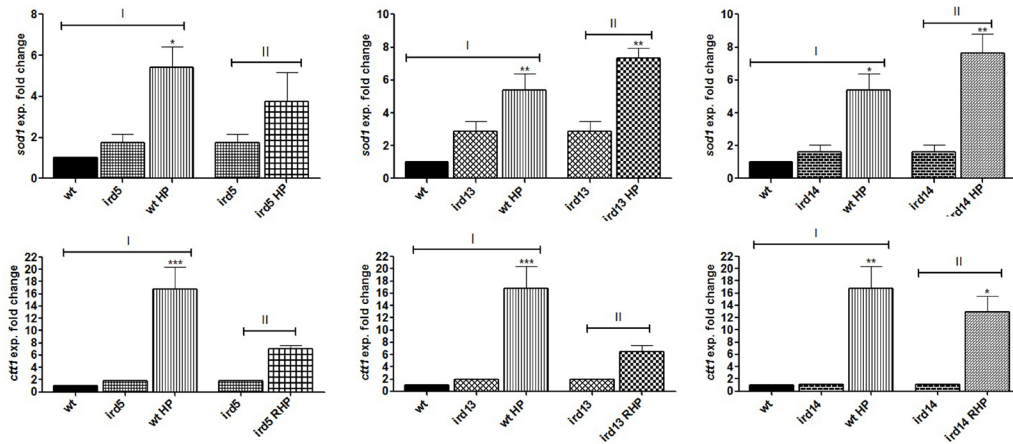

Figure 3. Expression levels of *ctt1* and *sod1* genes. The expression levels of *ctt1* and *sod1* genes in treated wild-type and *ird* mutants and untreated cells grown under repressed condition were determined according to Pfaffl method relative to the actin gene. Significant differences between **I.** untreated wild-type and untreated *ird* mutants and treated wild-type cells and **II.** untreated and treated *ird* mutants were evaluated by the Tukey test. ***P < 0.0001; **P < 0.001; *P < 0.05. wt = wild type; HP = treated with H_2O_2.
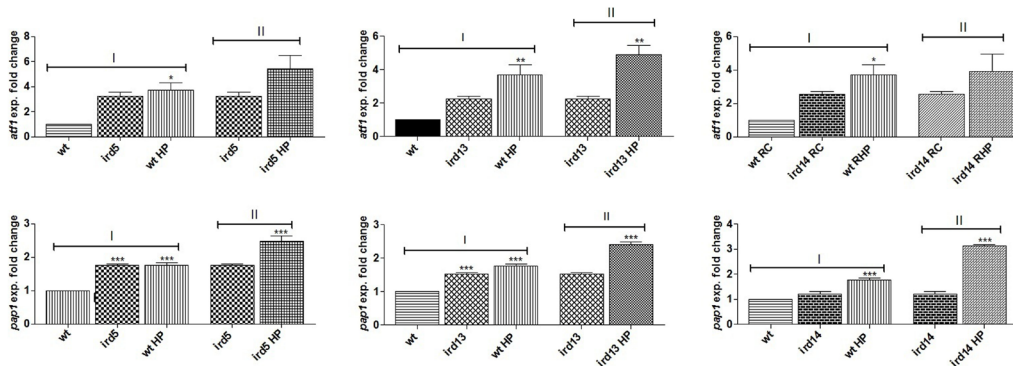

Figure 4. Expression levels of *atf1* and *pap1* genes. The expression levels of *atf1* and *pap1* genes in treated and untreated wild-type and *ird* mutant cells grown under repressed condition were determined according to Pfaffl method relative to the actin gene. Significant differences between **I.** untreated wild-type and untreated *ird* mutants and treated wild-type cells and **II.** untreated and treated *ird* mutants were evaluated by the Tukey test. ***P < 0.0001; **P < 0.001; *P < 0.05. wt = wild type; HP = treated with H_2O_2.

We compared the expression level of the *sty1* gene encoding the Sty1 protein kinase, which is an upstream regulator of the Atf1 and Pap1 transcription factors, in *S. pombe ird* mutants and wild-type cells (Ikner and Shiozaki, 2005). In this study, a significant increase was observed in *sty1* expression in the *ird5*, *ird13*, and *ird14* mutants under non-stressed condi-

tions (Figure 5). The expression level of *sty1* significantly increased in wild-type cells under stressed conditions. As shown in Figure 5, under the same conditions, the *sty1* expression level increased in the mutants with the exception of the *ird13* mutant. The phosphorylation level of the Sty1 protein was analyzed by immunoblotting with anti-phospho p38 (Thr[182]) in wild-type and *ird5*, *ird13*, and *ird14* mutant cells treated with H_2O_2 (Figure 6). With the exception of the *ird13* mutant, increased phosphorylation of Sty1-MAPK was observed in the *ird* mutants under non-stressed conditions, while the phosphorylation state increased dramatically in all *ird* mutants under stressed conditions. These findings suggest that the resistance of *ird* mutants to oxidative stress might result from the constitutive activation of *sty1* and/or increased phosphorylation of Sty1.
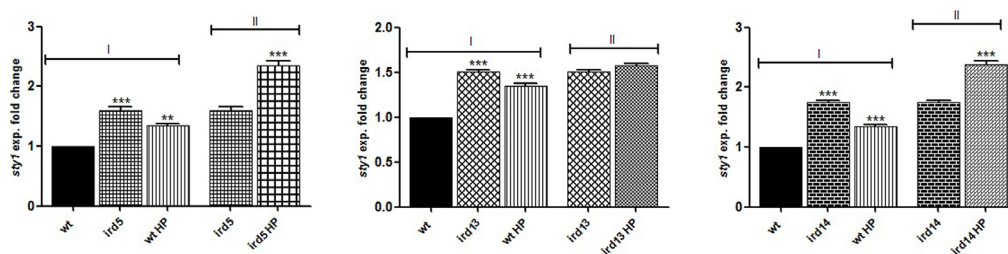
Figure 5. Expression levels of the *sty1* gene. The expression levels of the *sty1* gene in wild-type and *ird* mutants treated with H_2O_2 or untreated, after grown in rich media containing %3 glucose (repressed media), were determined according to Pfaffl method relative to the actin gene. Significant differences between **I.** untreated wild-type and untreated *ird* mutants and treated wild-type cells and **II.** untreated and treated *ird* mutants were evaluated by the Tukey test. ***$P < 0.0001$; **$P < 0.001$; *$P < 0.05$. wt = wild type; HP = treated with H_2O_2.
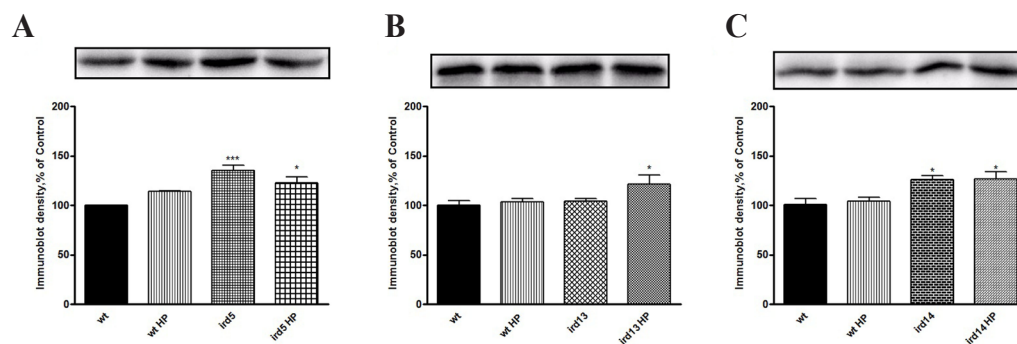
Figure 6. Detection of phosphorylated Sty1 by immunoblotting. The phosphorylated Sty1 protein was analyzed by immunoblotting with anti-phosphorylated p38 (Thr[182]) as described in "Experimental procedures" in wild-type and *ird* mutants treated with H_2O_2 or untreated, after grown in rich media containing 3% glucose (repressed media). **A.** *ird5*. **B.** *ird13*. **C.** *ird14*. All results were evaluated by the Tukey *t*-test. ***$P < 0.0001$; **$P < 0.001$; *$P < 0.05$. wt = wild type; HP = treated with H_2O_2.

CONCLUSIONS

As summarized in Table 1, the reduced glucose consumption efficiency of *ird* mutants might cause a lack of glucose repression in these mutants. The significantly increased

expression of *fbp1* in all *ird* mutants including *ird11* (Palabiyik et al., 2012) can therefore be interpreted as a lack of glucose repression in these mutants. Additionally, inefficient glucose uptake in *ird* mutants can cause an increased oxidative stress response in *S. pombe*. This may be explained by the fact that PKA activated by glucose starvation with the stress-activated MAPK pathway regulates the transcription of downstream genes via protein-DNA interactions at UAS1 and UAS2 of the *fbp1* gene (Neely and Hoffman, 2000).

Table 1. Summary of some studies concerning resistant to glucose repression mutants of *Schizosaccharomyces pombe*.

	Wild type	*ird5*	*ird13*	*ird14*	References
Glucose repression	91%	73%	41%	21%	Kig et al., 2005
Glucose consumption rate	87%	19%	37%	12%	Kig et al., 2005
Expression level of *fbp1* according to wild type	-	x8 up regulation	x4 up regulation	x4 up regulation	This study
Expression level of *hxk2* according to wild type	-	x2 up regulation	Down regulation	Down regulation	This study
Resistance to oxidative stress under repressed condition	20%	60%	75%	75%	This study

Finally, lowering the caloric intake by reduced glucose consumption efficiency of *ird* mutants might lead to resistance to oxidative stress in these mutants. However, there is a conflict in our results related to the expression of the *fbp1* and *hxk2* genes in the *ird5* mutant (like that of the *ird11* mutant; Palabiyik et al., 2012). Glucose detection in *S. pombe* occurs through a Git3 glucose-sensing receptor, which is encoded by the *git3* gene. This glucose receptor activity resembles the glucose signaling that is triggered by the cAMP-dependent PKA pathway (Welton and Hoffmann, 2000; Hoffman, 2005) and glucose utilization as an energy source that is started by glucose phosphorylation by the Hxk2 kinase enzyme, which is encoded by the *hxk2* gene. Taken together, this study implies that the deficiency of glucose repression in *ird* mutants might lead to resistance to oxidative stress in these mutants. In addition, glucose signaling mechanisms leading to glucose repression and glucose utilization as an energy source might be regulated apart from each other. Alternatively, like *S. cerevisiae*, *S. pombe* might have additional glucose detection systems. Our ongoing studies on this subject are important for understanding glucose sensing/signaling and glucose uptake in *S. pombe*. In this context, these mutants may be used as model systems for investigating glucose metabolism, glucose uptake, glucose sensing/signaling, and the oxidative stress response.

ACKNOWLEDGMENTS

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