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HSF1-mediated oxidative stress response to menadione in *Saccharomyces cerevisiae* KNU5377Y3 by using proteomic approach

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ABSTRACT

The hat shock transcription factor HSF1 in the yeast *Saccharomyces cerevisiae* regulates a wide range of genes and functions in diverse cellular reactions. To investigate the physiological response of HSF1 in the presence of menadione (MD) in *S. cerevisiae* KNU 5377Y3, wild-type (k3wt) and isogenic *hsf*1 mutant (k3h1) cells were introduced. HSF1 was induced when k3wt cells were exposed to the superoxide-generating agent MD and k3h1 cells were hypersensitive to MD. Under MD stress, k3h1 cells down-regulated the expression of metabolic enzymes (Hxk, Fba1, Pgk1, Eno2, and Adh1), antioxidant enzymes (Trx2 and porin), and molecular chaperones and their co-factors (Hsp104, Ssb1, Hsp60, Hsp42, Hsp26, Hsp12, Cpr1, and Sti1). In addition, k3h1 cells increased cellular hydroperoxide levels and protein carbonylation under MD stress as compared to k3wt cells. However, there was a moderate difference in the wild-type (b3wt) and mutant (b3h1) cells derived from *S. cerevisiae* S288C under the same conditions. Thus, these results show that HSF1 is an important component of the stress response system, acting as an activator of cell rescue genes in *S. cerevisiae* KNU5377Y3, and its expression protects the cells from MD-induced oxidative damage by maintaining redox homeostasis and proteostasis in the presence of MD.

Keywords: *Saccharomyces cerevisiae* KNU5377Y3; HSF1; Gene Expression; Menadione; Redox Homeostasis

1. INTRODUCTION

Oxidative stress caused via endogenous- and/or exogenous environmental changes can damage a wide range of cellular components such as carbohydrates, lipids, nucleic acids, and proteins. To protect or adapt against oxidative damage, cells have evolved a variety of cellular defense ystems, including the regulated expression of specific transcription factors. There are a number of transcriptional control systems that regulate the stress response in *Saccharomyces cerevisiae*, some of which are specific to one type of stress, or even a subset of stress responses, and some of which are more general. The main transcription factors involved in regulating the oxidative stress in yeast are Yap1, Skn7, and Msn2/4 [1-4]. Yap1 and Skn7 activate the expression of proteins that scavenge and destroy reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide anion (O_2^{*-}), and hydroxyl radical (HO^*). Msn2/4 controls the expression of a small number of antioxidants as well as heat shock proteins (Hsp), molecular chaperones, metabolic process enzymes, and members of the ubiquitin-proteasome degradation systems [5]. Some minor stress-related transcription factors in *S. cerevisiae* include Yap2p (Cad2p), which is turned on in response to toxic compounds; Yap4p (Cin5p/Hal6p) and Yap6p (Hal7p), which are turned on in response to osmotic stress; and Yap8p/Acr1p, which is turned on in response to arsenic stress [2].

HSF1 is a transcriptional activator that mediates eukaryotic gene expression in response to heat shock, pathogen infection, and inflammation, pharmacological agents, and other environmental stresses [6], thereby playing a central role in the regulation of cellular homeostasis [7]. Although genes encoding heat shock proteins and molecular chaperones are the best characterized targets of HSF1, recent genome-wide localization of *S. cerevisiae* HSF1 has revealed novel HSF1 targets involved in a wide range of cellular functions [3,7]. HSF1 activates multiple signaling pathways in response to oxidative stress; the degree to which a given pathway is activated is highly dependent on the nature and

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duration of the stress, as well as the cell type [3]. More recent work has extended these findings to suggest that HSF1 is actually linked to oxidative stress tolerance. When cells are exposed to the ROS generator menadione (2-methyl-1,4-naphtoquinone; MD), HSF1 increases the mRNA expression of *CUP*1, a gene encoding metallothionein [8] and *ERO*1, a gene encoding thiol oxidase, which is required for oxidative protein folding in the endoplasmic reticulum [5,9]. In mice, HSF1 knock-out caused an alteration of redox homeostasis and increased oxidative damage [1].

In a previous study, we identified that HSF1 of the *S. cerevisiae* strain KNU5377Y is induced in the presence of MD and activates a stress defense mechanism distinct from the mechanism induced when reference strain BY4741 (derived from S288C) was exposed to oxidants [10]. Among these oxidants, MD is a quinine compound that undergoes redox cycling. One-electron transfer, mainly from the mitochondrial respiratory chain, forms semiquinone radicals that can rapidly reduce O_2, thereby generating superoxide radical intracellularly and regenerating the quinine [4,10]. The current study was carried out to obtain further information on the relationship between HSF1 expression and stress response at the translational level in *S. cerevisiae* KNU5377Y3. Our results demonstrate that HSF1 expression in KNU 5377 Y3 leads to the activation of cell rescue genes via a mechanism different from that observed in BY4743 when cells were challenged with MD. Thus, the results obtained suggest another HSF-mediated stress responsive mechanism that is regulated by an oxidative-stress regulon, unlike previously studied systems in BY4743.

2. MATERIALS AND METHODS

2.1. Yeast Growth Conditions

Yeast cells were grown aerobically in a YPD medium (1% yeast extract, 2% peptone, and 2% dextrose) at 30˚C overnight, transferred to fresh YPD medium, and further cultured by shaking at 160 rpm [11]. Once a mid-log phase (OD_{600} = 2.0) was reached, cells were challenged directly with 0.4 mM MD (water-insoluble form; 2-methyl-1,4 naptoquinone; Sigma) for 1 h at 30˚C. Cells were harvested by centrifugation and the pellets were washed twice with a cold phosphate buffered saline (PBS), washed once with chilled distilled water, and then used for the subsequent experiments.

2.2. Stress Sensitivity to Menadione

For cell viability, mid-log phase cells were exposed to 0.0, 0.2, 0.4, 0.6, and 0.8 mM MD for 1 h, and then were diluted, spread on YPD agar plates, and incubated for 2 - 3 days. Cell viability was calculated by measuring colony-forming units (CFU), and the number of colonies grown in YPD liquid medium without MD treatment was set to 100%. For growth rate analysis, cells (1×10^6 cells per ml) were inoculated in YPD media and simultaneously treated with 45 μM MD. Optical density was measured at 600 nm at 2 h intervals for the indicated time. For the spotting assay, mid-log phase cells (OD_{600} = 2.0) were challenged with 0.4 mM MD at 30˚C for 1 h in YPD liquid media, and then diluted to 10^{-5}. An aliquot (5 μl) of each diluted sample was spotted onto YPD agar medium to monitor sensitivity. Wild-type (k3wt) and its isogenic Hsf1 mutant *hsf*1Δ (k3h1) cells, wild-type (b3wt) and *hsf*1Δ mutant (b3h1) cells from *S. cerevisiae* BY 4743, and wild-type (WT) and porin mutant (*por*1Δ) cells from *S. cerevisiae* BY4741 were used for stress sensitivity to MD.

2.3. Sequence Analysis

The mid-log phase cells grown for 6 h at 30˚C in a YPD liquid medium were harvested by centrifugation. Total RNA was extracted using the SV Total RNA Isolation system (Promega) according to the manufacturer's instructions. cDNA synthesis from total RNA was performed using an AccuPower RT Premix (Bioneer). The reaction mixture contained 0.1 μg of template RNA and 200 pmol of oligo dT_{18} primer in a total volume of 20 μl [12]. The reaction was conducted according to the manufacturer's instructions. The HSF1 coding region was amplified from the cDNA by PCR using *Taq* and *Pfu* polymerases (Roche) with the following primers: HSF1-A (5'-TGCAGTTCATGCATATTAAGTGAGT-3') and HSF1-D (5'-AGTCAATATAAGTACGCCAACTTGC-3'). PCR reaction conditions were as follows: initial denaturation at 94˚C for 3 min, followed by 30 cycles of 1 min at 94˚C, 1 min at 54˚C, 4 min at 72˚C, and a final extension of 10 min at 72˚C. The PCR product was extracted from 1.0% agarose gel using Wizard® SV Gel and PCR Clean-Up System (Promega) and inserted into the TOPO TA cloning vector (Invitrogen) according to manufacturer's protocol. The cloned plasmid was sequenced using the M13 primer set. The sequenced nucleotide was translated using European Molecular Biology Laboratory (EMBL)-European Bioinformatics Institute (EBI) Transeq (http://www.ebi.ac.uk/Tools/st/emboss_transeq/) software. Alignment of the predicted amino acid sequence was carried out using DAN Data Bank of Japan (DDBJ) ClustalW (http://clustalw.ddbj.nig.ac.jp/) software.

2.4. Western Blot Analysis

Cell lyates were prepared by lysing cells in a lysis buffer [50 mM Tris-HCl, pH 7.4, 5% glycerol, 2% sodium dodecyl sulfate (SDS), 1.5% β-mercaptoethanol, 1 mM

PMSF, and EDTA-free protease inhibitor cocktail (Roche)] and crude proteins were extracted using glass beads [13]. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the Laemmli method [14]. Denatured proteins (25 μg) were loaded into 10%, 12%, or 15% polyarylamide gels and then electro-phoretically transferred to a PVDF membrane (Bio-Rad). The PVDF membranes were incubated for 1.5 h at room temperature in a blocking buffer containing TBST (0.05% Tween-20, 10 mM Tris-HCl, pH 7.6, 150 mM NaCl), 5% non-fat skim milk, and 0.02% sodium azide. The blotted membranes were incubated overnight at 4˚C with the following primary antibodies: anti-Hsp104, and antiHsp60, (Stressgen), anti-glucose-6-phosphate dehydrogenase, and anti-Zpr1 (Sigma), anti-hexokinase (Abcam), anti-porin (Invitrogen), anti-tubulin (Millipore), and antiHsp82, anti-Ssa1, anti-Ssb1, anti-Hsp42, anti-Hsp26, anti-Hsp12, anti-Sti1, anti-Grp, and anti-Cpr1 [10] antibodies, After washing 4 times for 40 min with TBST, the membranes were incubated for 1.5 h at room temperature with anti-rabbit IgG (H + L) (Promega) or antimouse IgG (Millipore) secondary antibodies conjugated to HRP, washed 4 times for 40 min with TBST, and developed using enhanced chemiluminescence western blot detection kit (GE Healthcare).

2.5. Two-Dimensional Gel Electrophoresis and Protein Identification

The mid-log phase yeast cells were exposed to 0.4 mM MD for 1 h at 30˚C with shaking.

After washing twice with cold PBS, harvested cells were agitated 5 times for 5 min using a MicroMixer in a lysis buffer [80 mM Tris-HCl, pH 8.0, 2% SDS, 1.5% *β*-mercaptoethanol , 5% glycerol, 1 mM PMSF, and EDTA-free protease inhibitor cocktail (Roche)] and an equal amount of glass beads (400 - 600 micron; Sigma). Following centrifugation at 13,000 rpm for 20 min at 4˚C, the cleared supernatants were incubated with DNase/RNase/Mg mix on ice for 15 min before boiling for 5 min and cooling on ice for 5 min. Proteins in the supernatants were precipitated by trichloroacetic acid (final 10%) for 1 h on ice, centrifuged at 15,000 rpm for 20 min at 4˚C, and then washed 5 times with HPLC-grade ethanol containing 0.07% *β*-mercaptoethanol. The washed pellets were dried using a Speed Vac and were resuspended in sample buffer [9.5 M urea, 4% CHAPS, 0.1 M dethiothreitol (DTT), 40 mM Tris and 0.2% Bio-Lyte (3 - 10; Bio-Rad)] for 1 h and centrifuged at 15,000 rpm for 30 min. The cleared supernatants were transferred to a new tube, and the protein concentration was measured by a modified Bradford method [15] using a protein assay reagent (Bio-Rad). First-dimensional isoelectric focusing (IEF) was carried out at 20˚C on commercial immobilized pH gradient (IPG) strips (17 cm, pH 4 - 7; Bio-Rad), with a maximum current limitation of 50 μA/strip using Protean IEF Cell (Bio-Rad). The protein sample (1.0 mg) was loaded onto the bottom of an IPG strip. Running conditions were conducted according to the manufacturer's protocol. After equilibration following IEF, the strips were loaded on a 12% SDS-PAGE gel. Electrophoresis was performed on 1.0-mm thick gels at a constant current of 20 mA per gel at 18˚C. After electrophoresis, the gels were fixed, stained with Coomassie Brilliant Blue R-250 (Sigma) for 3 h, and then destained. Spots upregulated in wild type KNU 5377Y3 (k3wt) cells were excised for matrix associated laser-desorption ionization time-of-flight mass-spectrometry (MALDI-TOF MS) analysis. Protein identification was carried out using ProFound (http://prowl.Rockefeller.edu/profound_bin/WebProFound) software. The occurrence of a degraded protein species was assessed on the basis of the simultaneous occurrence of the following events: the definition of protein identification (Pro-Found's Est'd Z score > 1.6) and an evident discrepancy, with respect to the expected *Mr* values of the intact protein ($\Delta Mr > 30\%$).

2.6. Redox State and Protein Oxidation

The intracellular hydroperoxide level was spectrophotometrically determined by ferrous ion oxidation in the presence of a ferric ion indicator, xylenol orange (FOX) as described previously [10]. To measure the ROS levels, cells in the exponential growth phase were incubated for 20 min at 30˚C with 100 μM dichlorodihydrofluorescein diacetate (DCFHDA) and 100 μM rhodamine 123 (DHR 123), exposed to 0.4 mM or 20 mM H_2O_2 for 1 h with shaking, washed twice with PBS buffer, and then resuspended in the same buffer. Cells loaded with the fluorescent probes were imaged by fluorescence microscopy (excitation, 488 nm; emission, 525 nm). Carbonyl content was also measured by the spectrometric method reported previously [16].

2.7. Statistical Analysis

All biochemical experiments were carried out in at least 3 independent repetitions. Results are expressed as mean standard deviation (SD). The results of the spotting assay, growth kinetics, and redox state are representative of at least 2 independent experiments carried out under identical conditions.

3. RESULTS

3.1. Identification and Sequence Analysis of *S. cerevisiae* KNU5377Y3 HSF1

The complete sequence of HSF1 from diploid *S. cere-*

visiae KNU5377Y3 was analyzed. The complete HSF1 ORF encodes a protein consisting of 833-amino-acids, with a predicted molecular weight of approximately 93 kDa. The deduced amino acid sequence was 98.2% identical to that of the diploid *S. cerevisiae* S288C. Fifteen amino acids from KNU5377Y3 HSF1 were substituted as compared those of S288C HSF1: S72P, S123T, K125E, D139N, W254R, N283S, S305I, Q377K, G433A, E579D, K614E, W630L, N799K, K802E, and A831V (**Figure 1**). These results suggest that the genetic variation of the KNU5377Y3 HSF1 gene could explain why previous reports showed a different stress response in yeast cells exposed to unfavorable conditions.

3.2. HSF1 Upregulation Attenuates Stress Sensitivity and Cells Expressing Its Isogenic Mutant Are Hypersensitive to Menadione in Diploid *S. cerevisiae* KNU5377Y3

To examine whether HSF1 is activated under MD-induced oxidative stress in wild-type cells of *S. cerevisiae* KNU5377Y3 (k3wt), semi-quantitative RT-PCR and western blot were conducted. As shown in **Figure 2(a)**, Hsf1 expression in k3wt cells was highly induced at the translational level when yeast cells were exposed to 0.4 mM MD for 1 h. However, there was a distinct difference in Hsf1 expression in wild-type cells of *S. cerevisiae* BY4743 (b3wt) in the absence and presence of MD. To investigate if the Hsf1 activation in k3wt cells is involved in the stress response to MD, KNU5377Y3 cells expressing the isogenic Hsf1 mutant *hsf*1Δ (k3h1) were introduced. In addition, wild-type (b3wt) and *hsf*1Δ mutant (b3h1) cells from *S. cerevisiae* BY4743 were used as positive control cells. Each genotype used is described in **Table 1**. Under MD stress, the k3h1 cells were dose-dependently hypersensitive as compared to the k3wt cells. Although this notable distinction was not observed in both the b3wt and b3h1 cells, there were minor differences in both cells (**Figure 2(b)**). This difference in stress response to MD was strongly supported by growth kinetics and spotting assay. In the presence of 45 μM MD, the k3wt cells showed a faster recovery than the k3h1 cells over the indicated time. However, these significant differences were not found in the b3wt and b3h1 standard cells under the same conditions (**Figure 2(c)**). These observations were confirmed by the agar plate assay. The k3wt cells recovered rapidly in the presence of 0.4 mM MD as compared to the k3h1 cells, while cell survival was not affected in the b3wt and b3h1 cells under the same MD stress (**Figure 2(d)**). In contrast to most findings reported to date, our results indicate that HSF1 in *S. cerevisiae* KNU5377Y3 is induced by MD stress and is a critical component of the stress response.

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S288C       INPSLDPQSAASPVPSSSFFHDSRKPSTSTHLVRRGTPLGIYQTNLYGHNSRENTNPNST 120
KNU5377     INPSLDPQSAAPPVPSSSFFHDSRKPSTSTHLVRRGTPLGIYQTNLYGHNSRENTNPNST 120
            ************.************************************************

S288C       LLSSKLLAHPPVPYGQNPDLLQHAVYRAQPSSGTTNAQPRQTTRRYQSHKSRPAFVNKLW 180
KNU5377     LLTSELLAHPPVPYGQNPNLLQHAVYRAQPSSGTTNAQPRQTTRRYQSHKSRPAFVNKLW 180
            **:*:***************:********************************************

S288C       VKSGSIQSSSDDKWQFENENFIRGREDLLEKIIRQKGSSNNHNSPSGNGNPANGSNIPLD 300
KNU5377     VKSGSIQSSSDDKRQFENENFIRGREDLLEKIIRQKGSSNNHSSPSGNGNPANGSNIPLD 300
            ************** ****************************.******************

S288C       NAAGSNNSNNNISSSNSFFNNGHLLQGKTLRLMNEANLGDKNDVTAILGELEQIKYNQIA 360
KNU5377     NAAGINNSNNNISSSNSFFNNGHLLQGKTLRLMNEANLGDKNDVTAILGELEQIKYNQIA 360
            **** ************************************************************

S288C       ISKDLLRINKDNELLWQENMMARERHRTQQQALEKMFRFLTSIVPHLDPKMIMDGLGDPK 420
KNU5377     ISKDLLRINKDNELLWKENMMARERHRTQQQALEKMFRFLTSIVPHLDPKMIMDGLGDPK 420
            ******************:*********************************************

S288C       VNNEKLNSANNIGLNRDNTGTIDELKSNDSFINDDRNSFTNATTNARNNMSPNNDDNSID 480
KNU5377     VNNEKLNSANNIALNRDNTGTIDELKSNDSFINDDRNSFTNATTNARNNMSPNNDDNSID 480
            **************.************************************************

S288C       NRANSSTSSENPSLTPFDIESNNDRKISEIPFDDEEEEETDFRPFTSRDPNNQTSENTFD 600
KNU5377     NRANSSTSSENPSLTPFDIESNNDRKISEIPFDDEEEEDTDFRPFTSRDPNNQTSENTFD 600
            ****************************************:***********************

S288C       PNRFTMLSDDDLKKDSHTNDNKHNESDLFWDNVHRNIDEQDARLQNLENMVHILSPGYPN 660
KNU5377     PNRFTMLSDDDLKEDSHTNDNKHNESDLFLDNVHRNIDEQDARLQNLENMVHILSPGYPN 660
            ***************:***************** ******************************

S288C       LPSFNDHSYSTQADTAPENAKKRFVEEIPEPAIVEIQDPTEYNDHRLPKRAKK 833
KNU5377     LPSFNDHSYSTQADTAPEKAKERFVEEIPEPAIVEIQDPTEYNDHRLPKRVKK 833
            ******************:**:*****************************.**
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Figure 1. HSF1 sequence alignment of predicted amino acids between *S. cerevisiae* KNU5377 (KNU5377) and *S. cerevisiae* S288C (S288C). Red letters indicate no match. "*" indicates that the residues in that column are identical in all sequences in the alignment, ":" indicates the presence of conserved substitutions, and "." indicates the presence of semiconserved substitutions.

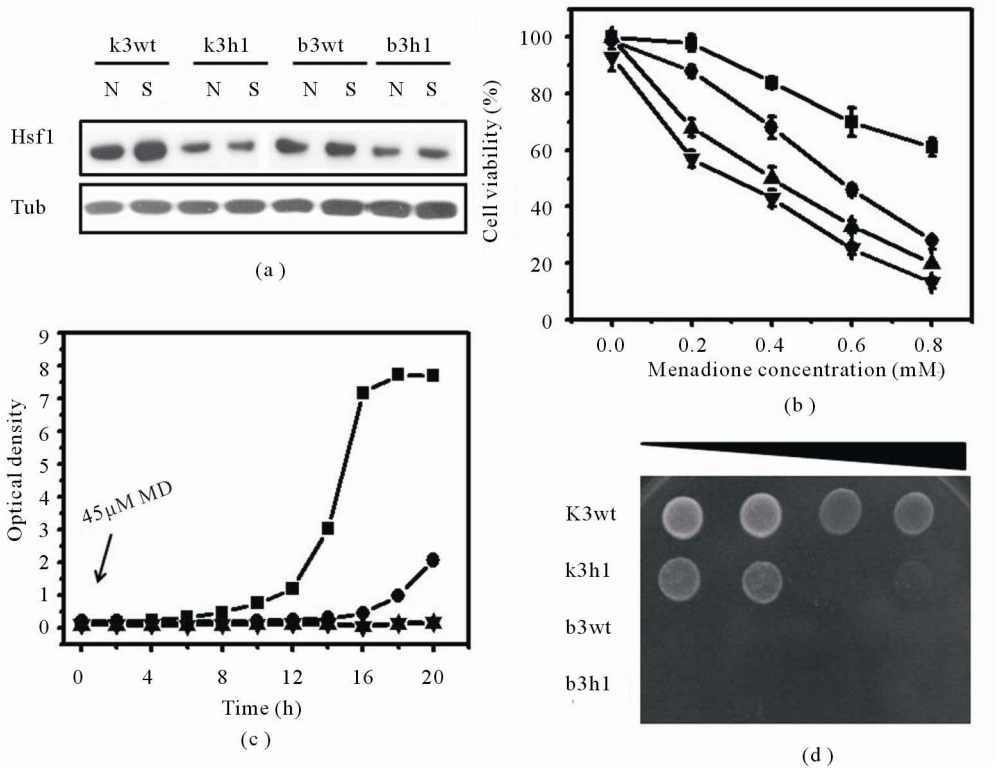

Figure 2. Expression changes of HSF1, growth kinetics, and cell survival in response to MD stress. (a) Translation analysis of Hsf1 in the wild-type and the *hsf*1Δ mutant cells from *S. cerevisiae* KNU5377Y3 and *S. cerevisiae* BY4743 in the absence (N) and presence (S) of 0.4 mM MD for 1 h by western blot. Tubulin (Tub) protein was used as a loading control. For cell survival assay, cell viability at different concentrations of MD (b), growth kinetics in the presence of 45 μM MD (c), and spotting assay using serial dilution (10^{-2} to 10^{-5}) after 0.4 mM MD for 1 h (d) were done as described in *Materials and Methods*. Square, k3wt cells; circle, k3h1 cells; upward triangle, b3wt cells; downward triangle, b3h1 cells.

Table 1. *S. cerevisiae* strains used in this study.

Strain	Genotype	Reference
BY4743 (b3wt)	*MATa*/*α*, *his*3Δ1/*his*3Δ1, *leu*Δ0/*leu*2Δ0, *lys*Δ0/*LYS*2, *MET*15/*met*15Δ0, *ura*3Δ0/*ura*3Δ0	EUROSCARF
BY4743 (b3h1)	*MATa*/*α*, *his*3Δ1/*his*3Δ1, *leu*Δ0/*leu*2Δ0, *lys*Δ0/*LYS*2, *MET*15/*met*15Δ0,*ura*3Δ0/*ura*3Δ0, *YGL*073*w*::*kanMX*4/*YGL*073*w*	EUROSCARF
KNU5377Y3 (k3wt)	*MATa*/*α*	11
KNU5377Y3 (k3h1)	*MATa*/*α*, *YGL*073*W*::*kanMX*4/*YGL*073*W*	11
BY4741 (WT)	*MATa*, *his*3Δ1, *leu*2Δ0, *met*15Δ0, *ura*3Δ0	EUROSCARF
*por*Δ1	*MATa*, *his*3Δ1, *leu*2Δ0, *met*15Δ0, *ura*3Δ0, *YNL*055*C*::*KanMX*4	EUROSCARF

*EUROSCARF: European *Saccharomyces cerevisiae* archives for functional analysis.

3.3. HSF1 Activates Cell Rescue Systems in Stress Response to Menadione in *S. cerevisiae* KNU5377Y3

HSF1 has been largely known as a transcription factor and several HSF-mediated stress responsive studies at the transcriptional level have been reported to date [4-7]. However, a survey of HSF1-controlled stress responses at the translational levels has been not conducted even though there can be a distinct difference between transcripts and proteins. Based on these facts, we further analyzed the effects of HSF1 during the stress response by using a proteomic approach. To achieve this, MS analysis following 2-D gel electrophoresis and western blot were performed. In the k3wt cells, MD treatment led to the accumulation of various proteins, including fruc-

tose-1,6-biphosphate aldolase (Fba1), heat shock protein 70 family (Hsp70; Ssa1), Hsp60p, enolase II (Eno2), 3-phosphoglycerate kinase (Pgk1), alpha subunit of the heteromeric nascent polypeptide-associated complex (Egd2), and alcohol dehydrogenase isoform 1 (Adh1), while the k3h1 cells down-regulated these proteins under the same conditions (**Figure 3**). To widely confirm HSF1-regulated target proteins, western blot was carried out. The k3wt cells had elevated expression of metabolic enzymes, including hexokinase (Hxk), and antioxidant enzymes including thioredoxin isoform 2 (Trx2) and porin (Por) under MD stress, while k3h1 cells down-regulated these proteins in the absence and presence of MD. Hexokinase and Porin expression in the b3wt and b3h1 cells increased in a HSF1-independent manner. However, Trx2 expression in b3h1 cells was repressed in the presence and absence of MD compared to that of the b3wt cells, even though the expression mildly increased under MD stress (**Figure 4(a)**). In particular, repression of porin protein increased stress sensitivity (**Figure 4(b)**, upper panel) and cytosolic and mitochondrial ROS levels (**Figure 4(b)**, lower panel) when yeast cells were treated with 0.2 mM MD for 1 h. In addition, the k3wt cells up-regulated molecular chaperones and their associated cofactors, including Hsp104, Hsp70 (Ssb1), Hsp60, Hsp 42, Hsp26, Cpr1, and Sti1 in the presence of MD, whereas k3h1 cells decreased expression of these proteins under the same stress condition. However, Hsp82, Grp, and Zpr1 expression in the k3h1 cells increased under MD stress. Although Hsp12 expression in the k3wt cells did not change, the expression in the k3h1 cells was reduced with and without MD treatment. The expression of most proteins, except Ssb1, increased in both the b3wt and b3h1 cells in the absence or presence of MD. The expression intensity of b3h1 cells was higher than that of b3wt cells under MD treatment. Ssb1 expression was reduced in the b3h1 cells under MD stress (**Figure 4(c)**). Therefore, our findings indicate that HSF1 in *S. cerevisiae* KNU5377Y3 cells regulates cell rescue systems through a mechanism different from the known regulation mechanism.

3.4. HSF1 Deficiency Causes Enhancement of Cellular ROS Levels and Protein Oxidation in *S. cerevisiae* KNU5377Y3

To investigate whether HSF1 affects cellular redox state in the presence of MD, cellular hydroperoxide levels and protein carbonyl contents were measured under MD stress. The level of intracellular hydroperoxides was evaluated by FOX assay and fluorescence microscopy with a cytosolic oxidant-sensitive probe DCFHDA. An intensity increase in DCF fluorescence was observed in both k3wt and k3h1 cells when they were exposed to MD, and this increase was more pronounced in the k3h1 cells. A similar result was detected in both the b3wt and b3h1 cells. Overall, the DCF fluorescence intensity increased from k3h1 to b3h1 to b3wt to k3wt cells in the presence of MD. In addition, we observed a moderate release of probe dye outside the k3h1 and b3h1 cells (**Figure 5(a)**). To confirm these findings, hydroperoxide levels using FOX reagent were also measured. Cellular hydroperoxide level in the k3h1 cells was 2-fold higher than that in the k3wt cells when yeast cells were exposed to MD, whereas a moderate increase was observed in the b3h1 cells under the same stress condition as compared to the b3wt cells. However, the hydroperoxide levels in both the b3wt and b3h1 cells were lower than those of the k3h1 cells under MD stress. There was a small increase in the ROS levels in the k3h1 cells under normal conditions as compared to the k3wt cells and the b3wt cells (**Figure 5(b)**). To determine whether increased ROS levels affect protein oxidation under MD stress, protein carbonyl contents were measured spectrophotometrically using dinitrophenylhydrazine (DNPH) in the absence and presence of MD. A significant increase in the carbonyl contents was observed in the k3h1 cells, with average levels approximately 1.5-fold higher than that in the k3wt cells during MD stress. An increase of the carbonyl contents in the k3h1 cells was detected under MD-free conditions. On the other hand, the carbonyl content of the b3h1 cells was slightly increased in the presence of MD as compared to that in the b3wt cells (**Figure 5(c)**). These results suggest that *hsf*1Δ deficiency in *S. cerevisiae* KNU5377Y3 increases protein oxidation following imbalance of the redox state.

4. DISCUSSION

HSF1 of diploid *S. cerevisiae* KNU5377Y3 displayed genetic variation of 15 amino acids compared to that of the diploid *S. cerevisiae* S288C (**Figure 1**). HSF1 expression in wild-type *S. cerevisiae* KNU5377Y3 (k3wt) cells was induced under MD stress and the isogenic *hsf*1Δ mutant (k3h1) cells were hypersensitive to MD. However, there was no phenotypic difference in the wild-type (b3wt) and *hsf*1Δ mutant (b3h1) cells of *S. cerevisiae* BY4743 derived *S. cerevisiae* S288C in the presence of MD (**Figure 2**). According to previous studies, most of HSF1 activation in yeast is induced by diamide, transition to the stationary phase, depletion of nitrogen or carbon sources, heat shock [17], and ROS generated by H_2O_2 treatment [4]. In *Drosophila*, a single HSF molecule responds directly to heat and to oxidative stress (H_2O_2) [18]. HSF1 of mammalian cells is essential for cell protection from stresses induced by heat shock and oxidative stress [19], or protection against acute cell injury, pharmaceutical agents, infection and inflammation [20]. In addition, HSF is essential for extended

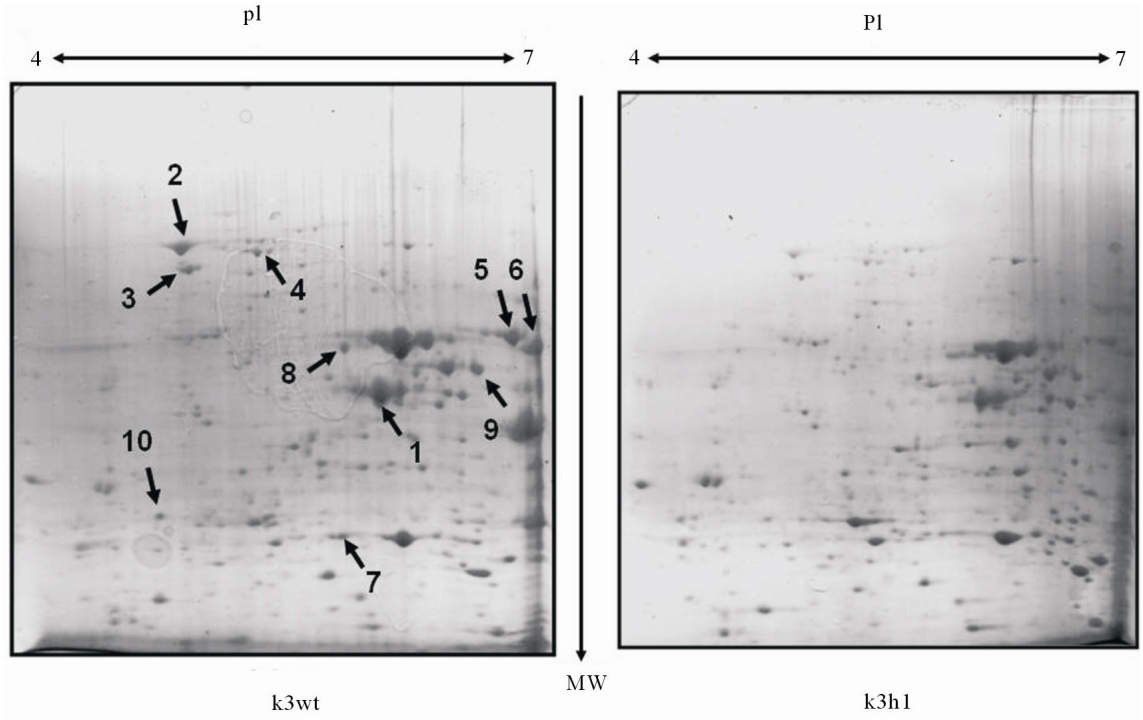

Figure 3. Comparative proteome analysis between k3wt cells and k3h1 cells after 0.4 mM MD treatment for 1 h. Protein (1 mg) was loaded on the IPG strip gel. After isoelectric focusing, equilibration, and SDS-PAGE (12%), the gels were stained with CBB R-250 and destained. Spots of interest were cut for MALDI-TOF MS analysis. Spots upregulated only in k3wt cells were arrowed and numbered. pI, isoelectric point; MW, molecular weight.

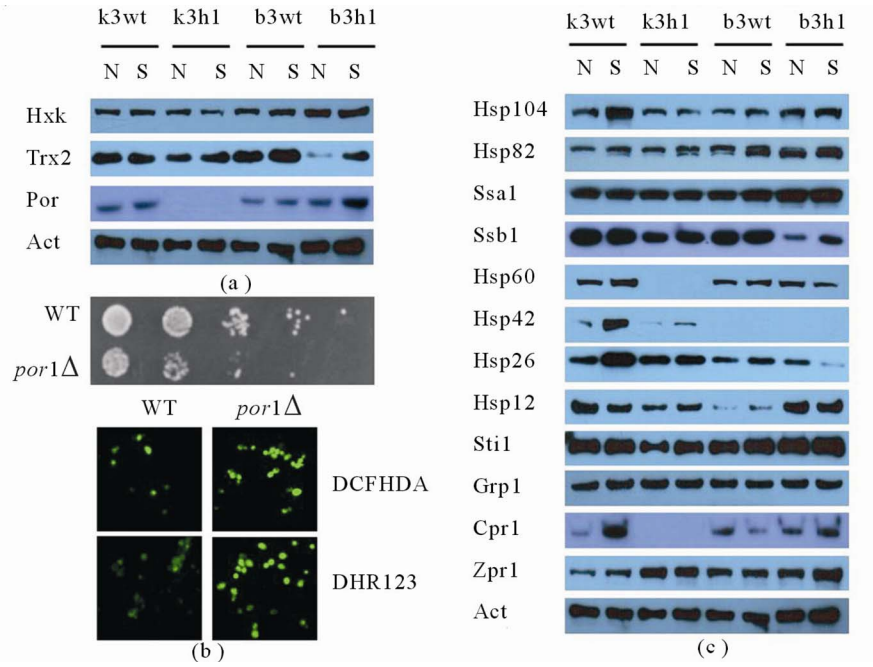

Figure 4. Expression regulation by HSF1 using western blot. (a) Expression changes of metabolic and antioxidant enzymes in the absence and presence of 0.4 mM MD; (b) Stress sensitivity (upper panel) and redox state (lower panel) of wild-type (WT) cells and *por*1Δ mutant (*por*1Δ) cells of *S. cerevisiae* BY4741 under 0.2 mM MD stress; (c) Expression analysis of molecular chaperones and their cofactors under 0.4 mM MD stress. Actin (Act) protein was used as a loading control for western blot. N, MD-untreated cells; S, MD-treated cells.

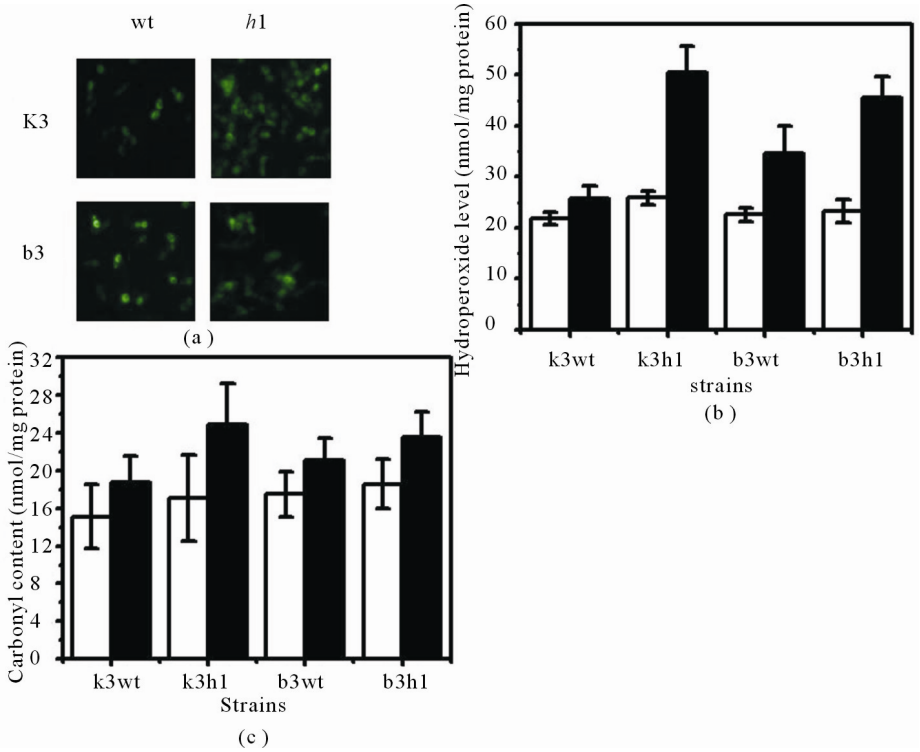

Figure 5. Measurement of cellular ROS level and carbonyl content under MD-induced oxidative stress. (a) Measurement of *in vivo* molecular oxidation. DCF fluorescence was measured in yeast cells exposed to MD for 1 h. Fluorescence images were obtained by fluorescence microscopy; (b) Cellular ROS level was measured by the spectrophotometric method using the FOX reagent; (c) Carbonyl contents were measured using the spectrophotometric assay. The levels are represented as nmol/mg protein. White bar, MD-untreated cells; black bar, MD-treated cells.

longevity in *Caenorhabditis elegans* [21], oogenesis, early larval development, survival of acute stress response in *Drosophila melanogaster* [22], and extraembryonic development and stress resistance in mice [17, 23]. *Escherichia coli* HSF (sigma32) provides stress tolerance to both high- and low-temperatures [24]. HSF in plants is required for thermotolerance [25] and stress tolerance to high-light stress and heat-shock, and H_2O_2 [26] in *Arabidopsis* and soybeans [27]. Taken together, unlike HSF1 of *S. cerevisiae* BY4743, our results indicate that HSF1 of *S. cerevisiae* KNU5377Y3 is induced by MD stress and is a key factor of intrinsic tolerance under MD-induced oxidative stress.

Under MD stress, k3wt cells up-regulated metabolic enzymes, including hexokinase (Hxk), fructose-1,6-biphosphate aldolase (Fba1), enolase II (Eno2), 3-phosphoglycerate kinase (Pgk1), and alcohol dehydrogenase isoform 1 (Adh1), and antioxidant enzymes, including thioredoxin 2 (Trx2) and porin (Por) in the presence of MD, while the k3h1 cells down-regulated these proteins (**Table 2**, **Figures 3** and **4(a)**). However, the expression of most proteins analyzed in the b3h1 cells was higher, except Trx2, as compared to the b3wt cells. Trx2 expression in the k3wt cells was HSF1-dependent. Hxk1 and Trx2 are involved in the stress response to menadione [28,29]. Deletion or downregulation of these antioxidant agents lead to a number of oxygen-dependent phenotypes, including oxygen sensitivity, slow growth, hypersensitivity to superoxide generating agents such as MD or paraquat, which are believed to accelerate aging, and auxotrophy for methionine and lysine [30]. 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 anions from the mitochondria to the cytosol [31], and its deletion increases stress sensitivity, following excess ROS accumulation in the cytosol and mitochondria (**Figure 4(b)**). In the k3h1 cells, the elevated ROS makes it difficult to modify neutralizing ROS or to repair oxidative damage (**Figure 5**), which is associated with sensitivity to MD-induced oxidative stress. Thus, our findings suggest that HSF1 in *S. cerevisiae* KNU5377Y3 cells regulates the expression of metabolic and antioxidant enzymes in response to MD stress.

The k3wt cells over-expressed molecular chaperones

Table 2. Identified proteins by MALDI-TOF MS analysis after 2-D PAGE.

Spot No.	Est'd Z	Protein information	%	pI	kDa
1	2.41	fructose 1,6-biphosphate aldolase	45	5.5	39.8
2	2.37	Ssa1p	52	5.0	69.8
3	2.40	Hsp60p	36	5.2	61.0
4	2.40	Ssb1p	36	5.3	66.7
5	2.41	Chain A, Mg-phosphonoacetohydroxymate complex of enolase	64	6.2	46.4
6	2.37	Pgk1p	60	7.1	44.7
7	2.35	Chain A, structure of triosephosphate isomerase	51	5.7	26.7
8	2.36	Eno2p	47	5.7	46.9
9	2.34	Adh1p	42	6.3	37.2
10	2.31	Egd2p	45	4.8	18.2

and their cofactors, including the alpha subunit of the heteromeric nascent polypeptide-associated complex (Egd2), Hsp104, Hsp70 (Ssb1), Hsp60, Hsp42, Hsp26, Cpr1, and Sti1, when the cells were challenged to MD stress, whereas the k3h1 cells down-regulated these proteins under the same conditions. However, Hsp82, Grp, and Zpr1 expression in the k3h1 cells increased greatly under MD stress as compared to the k3wt cells. In contrast, these results, except Ssb1, were not observed in the b3wt and b3h1 cells.

Most proteins expressed HSF1-independently under MD stress (**Figure 4(c)**). It has been known that the bulk of Hsps and some glycolytic enzymes such as phosphoglycerate kinase and enolase are induced by HSF1 during temperature shift [32,33]. Recently, it has been reported that HSF1 in yeast is able to activate the transcription of target genes (*CUP*1, *BTN*2, *SIS*1, *HSP*10, *SGT*2, and *SSA*3) in response to superoxide anion-generating agents menadione and potassium superoxide (KO_2) and the thiol-oxidizing compound diamide, as well as in response to heat shock [5]. In this study, we identified several new HSF1 target genes, including *Egd*2, *Hsp*104, *Ssb*1, *Hsp*60, *Hsp*42, *Hsp*26, *Hsp*12, *Cpr*1, and *Sti*1 under MD stress in *S. cerevisiae* KNU 5377Y3. Molecular chaperones are known to be involved in many cellular processes and pathways such as protein translocation across membranes, ribosomal RNA processing, and ER-associated protein degradation [34]. Chaperones that participate broadly in protein refolding, such as Hsp70 family (Ssa and Ssb), Hsp90, Hsp104, and sHsps (Hsp40, Hsp26, and Hsp12), promote the folding process through cycles of substrate binding and by cofactor proteins [35]. Hsp104 and Hsp70 act together with Hsp26 in protein refolding after stress-induced unfolding [36]. In addition, Hsp90 depends on its association with a variety of cochaperones and cofactors. The co-chaperones include Hsp70 and Hsp40 (Hsp42) [37], and cyclophilins Cpr6 and Cpr7 [38]. The formation of a complex between Hsp70 and Hsp90 is mediated through the association of both chaperones with an adaptor protein termed as an activator of the Ssa protein Sti1 [39] and an essential ER chaperone Grp (Grp94) that functions as an interaction domain of Hsp90 [10,40]. An imbalance of chaperone machinery systems in the k3h1 cells creates a difficulty in protein (re)folding under unstressed and stressed conditions when they are accompanied by elevated protein oxidation (**Figure 5(c)**). Protein carbonylation is an irreversible and irreparable modification. The *hsf*1 mutant yeast cells defective in transcriptional activation were sensitive to MD and diamide [5]. In mice, *hsf*1 knock-out alters cardiac redox homeostasis and increases oxidative damage via elevated superoxide anion production [1]. *HSF*-knockout cells attenuate cellular processes such as normal growth, activation of *HSP* genes in response to stress, embryonic development, inflammatory responses, fertility, and resistance to stress-induced apoptosis [1,20,32]. In addition, *HSF*-knockout in flies and worms affects normal growth and development, aging, and stress resistance [10,32]. To sum up our results, HSF1 in *S. cerevisiae* KNU5377Y3 cells regulates gene expression of various molecular chaperones which promote protein folding/refolding under MD stress by minimizing MD-induced oxidative damage, leading to a better stress response.

In conclusion, our results suggest that the discovery of HSF-mediated target genes at the translational level could be an effective approach because protein expression in the k3h1 cells is down-regulated under normal conditions. The identification of novel HSF1 target genes during MD stress reveals a more diverse function for

HSF1 than previously reported, suggesting that HSF1 affects the gene expression of various cell rescue systems, including molecular chaperones, antioxidant enzymes, and carbohydrate metabolism, playing an important role in oxidative stress and development by MD.

5. ACKNOWLEDGEMENTS

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