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The flavoprotein Tah18-dependent NO synthesis confers high-temperature stress tolerance on yeast cells

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ABSTRACT

Nitric oxide (NO) is a ubiquitous signaling molecule involved in the regulation of a large number of cellular functions. In the unicellular eukaryote yeast, NO may be involved in stress response pathways, but its role is poorly understood due to the lack of mammalian NO synthase (NOS) orthologues. Previously, we have proposed the oxidative stress-induced L-arginine synthesis and its physiological role under stress conditions in yeast *Saccharomyces cerevisiae*. Here, our experimental results indicated that increased conversion of L-proline into L-arginine led to NO production in response to elevated temperature. We also showed that the flavoprotein Tah18, which was previously reported to transfer electrons to the Fe–S cluster protein Dre2, was involved in NO synthesis in yeast. Gene knockdown analysis demonstrated that Tah18-dependent NO synthesis confers high-temperature stress tolerance on yeast cells. As it appears that such a unique cell protection mechanism is specific to yeasts and fungi, it represents a promising target for antifungal activity.

1. Introduction

Nitric oxide (NO) is a ubiquitous signaling molecule involved in the regulation of a large number of cellular functions. NO confers oxidative stress tolerance by enhancing cellular antioxidative activity in mammals and plants [1,2]. In mammals, L-arginine is converted into NO and L-citrulline in the presence of oxygen and NADPH by NO synthases (NOSs, EC 1.14.13.39). In mammal and yeast, cytochrome c oxidase can utilize nitrite instead of oxygen as an electron acceptor in hypoxia, leading to NO release [3]. In contrast, nitrite reductase (EC 1.7.2.1) is also an NO-forming enzyme that catalyzes the reduction of nitrite in some Gram-negative bacteria [4]. Bacterial nitrite reductase normally catalyzes a six-electron reduction of nitrite to ammonia, but this enzyme produces NO as a byproduct [5]. In addition, nitrite is chemically reduced to NO under the acidic and reducing conditions [6]. NO plays an important role in mammalian cells mainly through the cGMP-mediated signaling pathways by activating soluble guanylate cyclase (sGC) [7] and through posttranslational activation of proteins via *S*-nitrosylation [8].

The unicellular eukaryote yeast cells are used to study the relationship between NO and the neurodegenerative disease [9]. In *Saccharomyces cerevisiae*, low levels of NO may be involved in various stress response pathways including responses to high hydrostatic pressure, copper metabolism, and H_2O_2-induced apoptosis [10–12]. *S. cerevisiae* cells appear to produce NO by the mechanisms similar to those involved in classical NOS activity [10,13], and cross-reactive proteins with mammalian NOS antibodies have been detected in *S. cerevisiae* [11,13]. However, the synthetic mechanism and the physiological role of NO in *S. cerevisiae* remain unclear due to the lack of mammalian and bacterial NOS orthologues in the genome.

S. cerevisiae Σ1278b strain has the *MPR1* and its paralog (*MPR2*) genes, encoding the *N*-acetyltransferase Mpr1 that acetylates the proline metabolism intermediate, L-Δ^1-pyrroline-5-carboxylate (P5C)/L-glutamate-γ-semialdehyde (GSA) [14]. Gene disruption analysis revealed that Mpr1 converts P5C/GSA into *N*-acetyl-GSA for L-arginine synthesis in the mitochondria, indicating that Mpr1 mediates the L-proline and L-arginine metabolic pathways [15] (Fig. S1). Mpr1 was previously shown to protect yeast cells by reducing the intracellular reactive oxygen species (ROS) levels under various oxidative stress conditions [14,16,17]. With exposure to high-temperature, which causes oxidative stress via ROS generation in the mitochondria [18,19], the transcription of *MPR1* and *PUT1* was strongly induced, leading to an increase in intracellular L-arginine level [15]. We also obtained evidence that increased conversion of L-proline into L-arginine confers oxidative stress tolerance on yeast cells, indicating a novel antioxidative mechanism involved in stress-induced L-arginine synthesis requiring Mpr1 and Put1 [15].

Here, we report that increased conversion of L-proline into L-arginine led to NO production. Furthermore, we found that the Tah18 protein was involved in NO synthesis in *S. cerevisiae*, which

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lacks mammalian NOS orthologues in the genome. We also obtained evidence indicating that Tah18-dependent NO synthesis contributes to high-temperature stress tolerance in *S. cerevisiae* cells.

2. Materials and methods

2.1. NO detection

Free intracellular NO was detected with diaminofluorescein-FM diacetate (DAF-FM DA) (Sekisui Medical). Exponential yeast cells were incubated at 25 °C for 1 h in SD or SG medium containing 15 μM DAF-FM DA in the dark and then exposed to 100 μM L-arginine for 1 h or 39 °C for 4 h. The harvested cells were washed twice with 50 mM potassium phosphate buffer (pH 7.4), resuspended in 500 μl of distilled water and incubated at 25 °C for 30 min. For the visualization, cells were viewed under an Axiovert 200 M microscope (Carl Zeiss) with a 100× oil immersion objective and images were captured with an AxioCam MRm CCD camera (Carl Zeiss). For a quantitative analysis of NO production, the harvested cells were washed twice with 50 mM potassium phosphate buffer (pH 7.4), resuspended in 500 μl of distilled water and disrupted with glass beads in a Multi-Beads Shocker (Yasui Kikai). The fluorescence was measured with λ_{EX} = 500 nm and λ_{EM} = 515 nm using a fluorescence spectrophotometer (F-7000; Hitachi) and was normalized by protein in the mixture. An NO standard curve was established by determining the fluorescence of DAF-FM (Sekisui Medical) in the presence of various concentrations of 3-(2-hydroxy-1-methyl-2-nitroso-hydrazino)-*N*-methyl-1-propanamine (NOC7) (half-life of 5 min). The values are the means and standard deviations of three independent experiments. In the *in vitro* experiments, NO was detected with DAF-FM. The fluorescence from DAF-FM T, which is the reaction product of DAF-FM with NO, was measured with a fluorescence spectrophotometer. The excitation and emission wavelength for DAF-FM T were 500 and 515 nm, respectively.

2.2. NOS activity

S. cerevisaie cells were cultured to the stationary growth phase in YPD, SD, or SG medium at 25 °C. *Escherichia coli* strains BL21 (DE3) transformed with pET-DEST42 containing each ORF for screening of the NOS gene were grown at 37 °C in M9CA medium. When absorbance at 600 nm reached 0.6, isopropyl-β-D-thiogalactopyranoside was added to the culture medium to a final concentration of 1 mM to induce gene expression, and the cells were cultivated for 8 h at 25 °C. Both *S. cerevisaie* and *E. coli* cells were harvested, suspended in ice-cold buffer A [20 mM Tris–HCl (pH 7.4), 2 μM BH_4 and 1 mM DTT], and disrupted with glass beads in a Multi-Beads Shocker under cooling. The supernatants after centrifugation was precipitated with ammonium sulfate (70% saturation) and dialyzed with buffer A. The dialysates were used as enzyme sources. The NOS activity was assayed by a fluorescent method that measures nitrite formed nonenzymatically from NO [20]. The reaction mixture contained the following in a final volume of 80 μl: 5 mM L-arginine, 1 mM NADPH, 2 μM FAD, 2 μM FMN, 10 μM tetrahydrobiopterin, 2 mM $CaCl_2$, 37.5 μg/ml calmodulin, 150 μM DTT and the crude extracts (2–20 μg protein) in 50 mM Tris–HCl (pH7.4). After incubation at 30 °C for 60 min, nitrite formed nonenzymatically from NO after NOS activity was measured using a NO_2/NO_3 Assay Kit-FX (Dojindo). This method is based on the reaction of 2,3-diaminonaphthalene with nitrite under acidic conditions to form 1-naphthotriazole, a fluorescent product.

2.3. Strains, plasmids, culture media, plasmids construction, stress tolerance test, L-citrulline determination, Western blot analysis, real-time quantitative PCR analysis, sulfite reductase activity, and nucleotide sequence accession numbers

Details are described in Supplementary Materials and methods.

3. Results

3.1. Relationship between L-arginine-dependent NO generation and stress tolerance in S. cerevisiae

By H_2O_2 treatment into yeast cells, there was a twofold increase in L-arginine concentration, which might be crucial for NO production upon apoptosis [10], but the question remained whether high-temperature treatment that elevates ROS levels [15] might induce the endogenous production of NO via L-arginine in yeast cells. We attempted to detect NO in cells exposed to a high-temperature (39 °C) using the NO-specific fluorescent probe, DAF-FM DA (Fig. 1A). A quantitative analysis of NO production was also performed in the presence of a non-enzymatic NO donor, NOC7 (Fig. 1B). After incubation of the cells with L-arginine, NO production was clearly detected in all cells tested. Additionally, L-arginine-derived NO production was inhibited by pre-incubation of cells with a non-metabolized L-arginine analogue, N^G-nitro-L-arginine methyl ester (NAME), which is a NOS inhibitor. Interestingly, when yeast cells were exposed to 39 °C for 4 h, a high percentage of L5685 (wild-type) cells were shown to produce NO, whereas little NO was observed in LD1014 (*Δmpr1Δmpr2*) or L5685Dput1 (*Δput1*) cells. We also found that pre-treatment with NAME inhibited NO synthesis in L5685 cells.

We next examined whether nitrite is a source of NO in *S. cerevisiae* cells. The *rho*^{0} strain L5685rho^0, which lacks cytochrome c oxidase-dependent NO synthesis [3], clearly showed L-arginine-derived NO production (Fig. 1A). However, little NO was detected after exposure to high-temperature. This result is probably because *rho*^{0} cells fail to express proline oxidase activity, which requires the mitochondrial respiratory chain as an electron flow. We confirmed that the *rho*^{0} strains derived from the wild-type and *Δmpr1 Δmpr2* strains could not utilize L-proline as the sole nitrogen source, indicating that the *rho*^{0} strain is deficient in proline oxidase activity. Sulfite/Nitrite reductase involved in the reduction of nitrite to ammonia consists of the Met5 and Met10 complex, and the gene disruption results in deficiency in nitrite reductase activity [21,22]. L-Arginine- and high-temperature-induced NO production occurred in the *met5*- and *met10*-disrupted strains L5685Dmet5 and L5685Dmet10, respectively (Fig. 1A). These results suggest that NO is synthesized from L-arginine in yeast cells in response to incubation at 39 °C.

The mutants deficient in stress-induced L-arginine synthesis (LD1014 and L5685Dput1) showed a significant decrease in the survival rate after high-temperature treatment, as compared with that of wild-type cells (L5685) [15]. To investigate whether L-arginine-dependent NO synthesis contributes to stress tolerance, we tested the cell viability after exposure to 39 °C for 14 h under various conditions. When NAME or a NO scavenger, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) was added, L5685 cells were more sensitive to high-temperature than were the untreated cells (Fig. 2A). However, pre-treatment with NAME or cPTIO did not affect the survival rate of either LD1014 (Fig. 2B) or L5685Dput1 cells (Fig. 2C). These results demonstrate that NO synthesis is important for stress tolerance in cells in which L-arginine synthesis is induced under a high-temperature stress condition. Even in the presence of L-arginine, the viability of NAME-treated cells was significantly lower than that of untreated

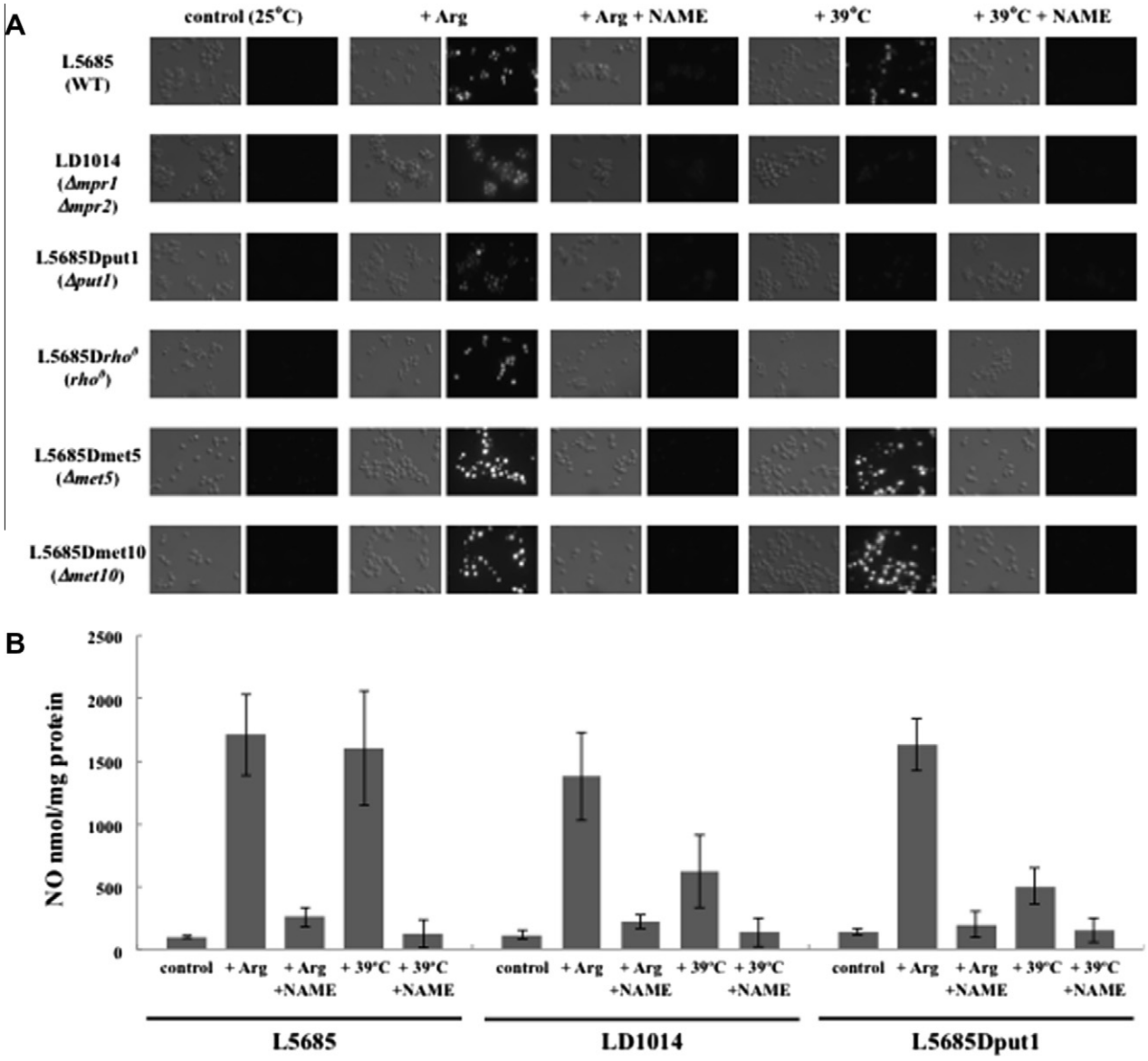

Fig. 1. NO production in yeast cells. (A) NO production was shown as fluorescence from DAF-FM DA in L5685 (WT), LD1014 (*Δmpr1Δmpr2*), L5685Dput1 (*Δput1*), L5685rho^0 (*rho*^{0}), L5685Dmet5 (*Δmet5*), and L5685Dmet10 (*Δmet10*) cells exposed to 100 μM L-arginine for 1 h (Arg) or 39 °C for 4 h (39 °C), with or without preincubation of 100 mM NAME (right panels). Cell morphology was observed through differential interference contrast (left panels). (B) NO production was quantitatively analyzed in L5685 (WT), LD1014 (*Δmpr1Δmpr2*), and L5685Dput1 (*Δput1*) cells exposed to 100 μM L-arginine for 1 h (Arg) or 39 °C for 4 h (39 °C), with or without preincubation of 100 mM NAME. An NO standard curve was established by determining the fluorescence of DAF-FM in the presence of various concentrations of NOC7. The values are the means and standard deviations of three independent experiments.

cells, probably because pre-incubation with NAME inhibited NO synthesis.

We next treated cells with a non-enzymatic NO donor, *S*-nitroso-*N*-acetylpenicillamine (SNAP). The viability of L5685 cells treated with SNAP remained virtually unchanged from that of untreated cells under a high-temperature stress condition (Fig. 2A). In contrast, the treatment with SNAP dramatically increased the survival rate of LD1014 (Fig. 2B) and L5685Dput1 cells (Fig. 2C). Interestingly, the positive influence of SNAP on stress tolerance was observed even in the presence of NAME. In contrast, cPTIO suppressed the positive effect of SNAP on LD1014 and L5685Dput1 cells. To exclude the possibility that SNAP triggers *S*-transnitrosation, we also treated cells with 1-hydroxy-2-oxo-3-(*N*-ethyl-2-aminoethyl)-3-ethyl-1-triazene (NOC12), which is a diazeniumdiolate NO donor without *S*-transnitrosations [23]. The treatment with NOC12 conferred stress tolerance on LD1014 and L5685Dput1 cells, as the same level as did SNAP (Fig. 2). These results suggest that NO production confers stress tolerance on yeast cells.

3.2. Identification of the protein involved in NO synthesis in *S. cerevisiae*

Given that NO is rapidly oxidized to nitrite, we performed an indirect measurement of NOS activity by monitoring nitrite formation. Considerable NOS activity was detected in the soluble extract of *S. cerevisiae* cells (Fig. 3A), in agreement with the results of previous studies [10,13], and this activity was L-arginine and NADPH-dependent, as was that of the mammalian NOSs. NOS activity was also detected in the soluble extract of *rho*^{0}, *Δmet5*, and *Δmet10* cells, suggesting that yeast NOS activity is independent on nitrite reductase (Fig. 3B). To identify the yeast protein involved in NO

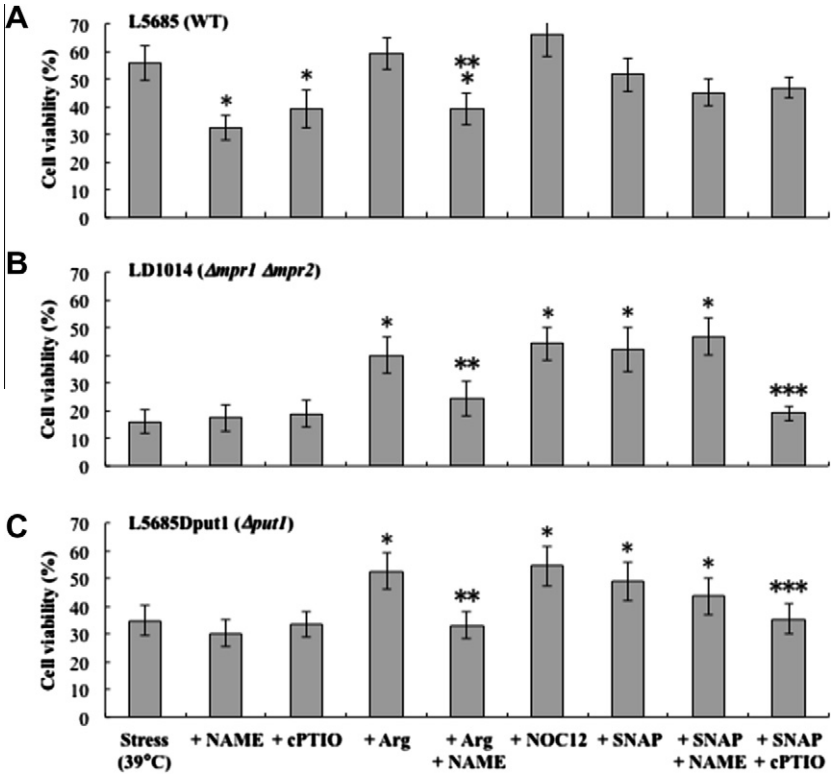

Fig. 2. NO-dependent stress tolerance in yeast cells. Cell viability was tested in L5685 (WT) (A), LD1014 (*Δmpr1Δmpr2*) (B), and L5685Dput1 (*Δput1*) (C) after exposure to 39 °C for 14 h in the presence of 1 mM cPTIO, 100 μM L-arginine (Arg), 40 μM NOC12, 62.5 μM SNAP, or/and 100 mM NAME. The values are the means and standard deviations of nine independent experiments. $^{*}p < 0.05$ vs. Stress (39 °C); $^{**}p < 0.05$ vs. + Arg; $^{***}p < 0.05$ vs. + SNAP by Student's *t* test.

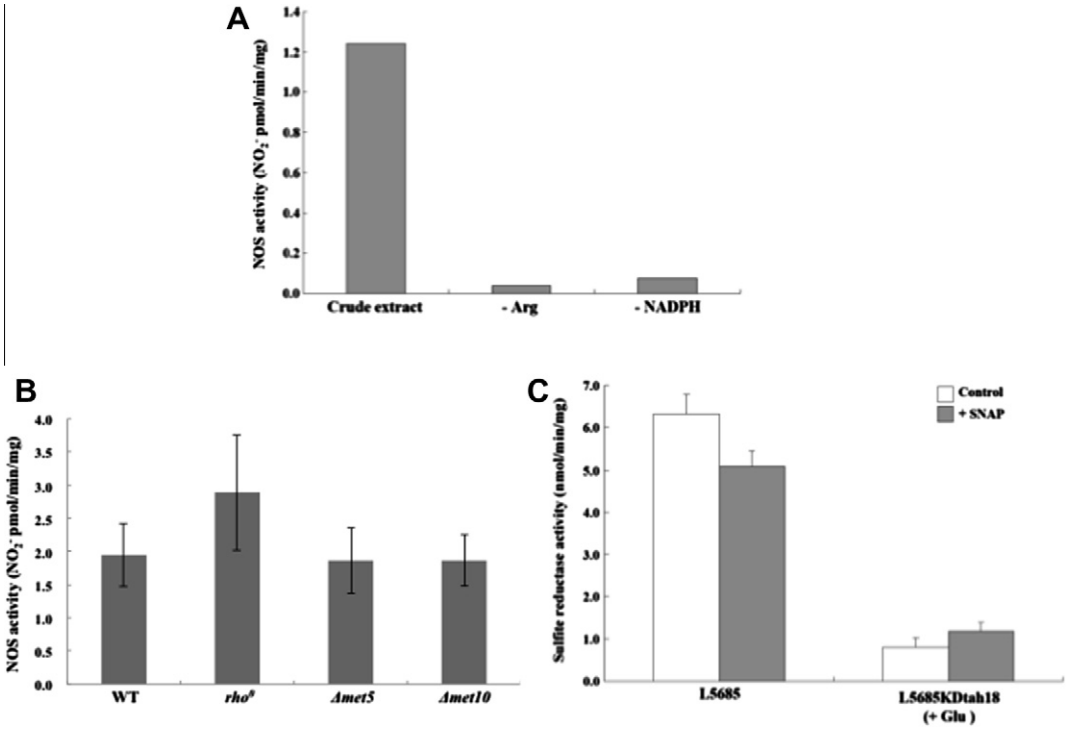

Fig. 3. NOS and sulfite reductase activities in the soluble extract of yeast cells. (A) The NOS activity in L5685 cells was measured by a fluorescent method that determines nitrite formed nonenzymaically from NO [22]. L-Arginine (Arg) or NADPH was omitted from the reaction mixture and replaced with 50 mM Tris–HCl buffer (pH 7.4). L5685 cells were cultured to the stationary growth phase in YPD medium at 25 °C. (B) The NOS activity was measured in L5685 (WT), L5685 *rho*^{0} (*rho*^{0}), L5685Dmet5 (*Δmet5*), and L5685Dmet10 (*Δmet10*) cells. Each strain was cultured to the stationary growth phase in SD medium at 25 °C. (C) Sulfite reductase activity was measured by using soluble extracts of L5685 and L5685KDtah18 cells pretreated with (gray bar) or without (white bar) 62.5 μM SNAP for 8 h. L5685KDtah18 cells were cultured in SD medium (+Glu) to suppress the expression of *TAH18*. The values are the means and standard deviations of three independent experiments.

synthesis, we focused on the putative oxidoreductase genes (NADH- or NADPH-dependent) found in the *S. cerevisiae* genome from the Saccharomyces Genome Database, based on the fact that mammalian NOSs require an NADPH-dependent oxidoreductase domain [24].

Of these 275 genes, 27 uncharacterized genes were expressed in *E. coli*. As a result, a significant NOS activity was detected only in the extract from cells expressing *TAH18* (Fig. S2). The Tah18 protein was previously shown to regulate an oxidative stress-induced cell death [25]. Netz et al. [26] also reported that the complex of Tah18 and the Fe–S cluster assembly protein Dre2 plays important roles in the cytosolic Fe–S protein biogenesis. Tah18 is a molecular partner of Dre2 but the previous work did not assay NOS activity of Tah18 [26]. We cannot exclude the possibilities that the *E. coli* NO producing system are enhanced by expressing *TAH18* and *E. coli* contaminations are responsible for the NOS activity as part of the enzyme, although NAME inhibited NOS activity of *TAH18*-expressing cells (154 or 76 pmol NO_2^-/min/mg in the absence or presence of 50 mM NAME, respectively).

3.3. Tah18-dependent NO production and its physiological role in *S. cerevisiae*

For functional analysis of Tah18, which is an essential protein for cell survival, we constructed the conditional knockdown strain L5685KDtah18, which expresses *TAH18* under the control of the galactose-inducible *GAL1* promoter. We observed little expression of *TAH18* and a very low level of NOS activity in L5685KDtah18 cells as compared with L5685 cells on glucose-containing medium, but the addition of galactose clearly induced *TAH18* expression and NOS activity (Fig. 4A). Strain TAH18-OE, which overexpresses *TAH18* under the control of the constitutive *TDH3* promoter, showed a higher level of NOS activity than that of L5685 (Fig. 4A). These results indicate that yeast NOS activity is dependent on Tah18.

Next, we examined whether Tah18 is involved in stress-induced NO synthesis (Fig. 4B). A quantitative analysis of NO production was performed in the presence of NOC7 (Fig. 4C). A high percentage of L5685 cells were shown to produce NO by the addition of L-arginine or exposure to 39 °C. In contrast, little NO production was observed in L5685KDtah18 cells grown in glucose-containing medium. Galactose dramatically induced NO production in L5685KDtah18 cells as well as NOS activity. This result indicates that L-arginine- and stress-induced NO synthesis is Tah18-dependent.

Finally, we investigated the effect of Tah18-induced NO production on stress tolerance. Real-time quantitative PCR analysis showed that the transcription of *TAH18* was induced after exposure to 39 °C or 0.5 mM H_2O_2 in L5685 cells (4.2 ± 0.9- and 2.7 ± 0.5-fold, respectively). Under the high-temperature stress condition,

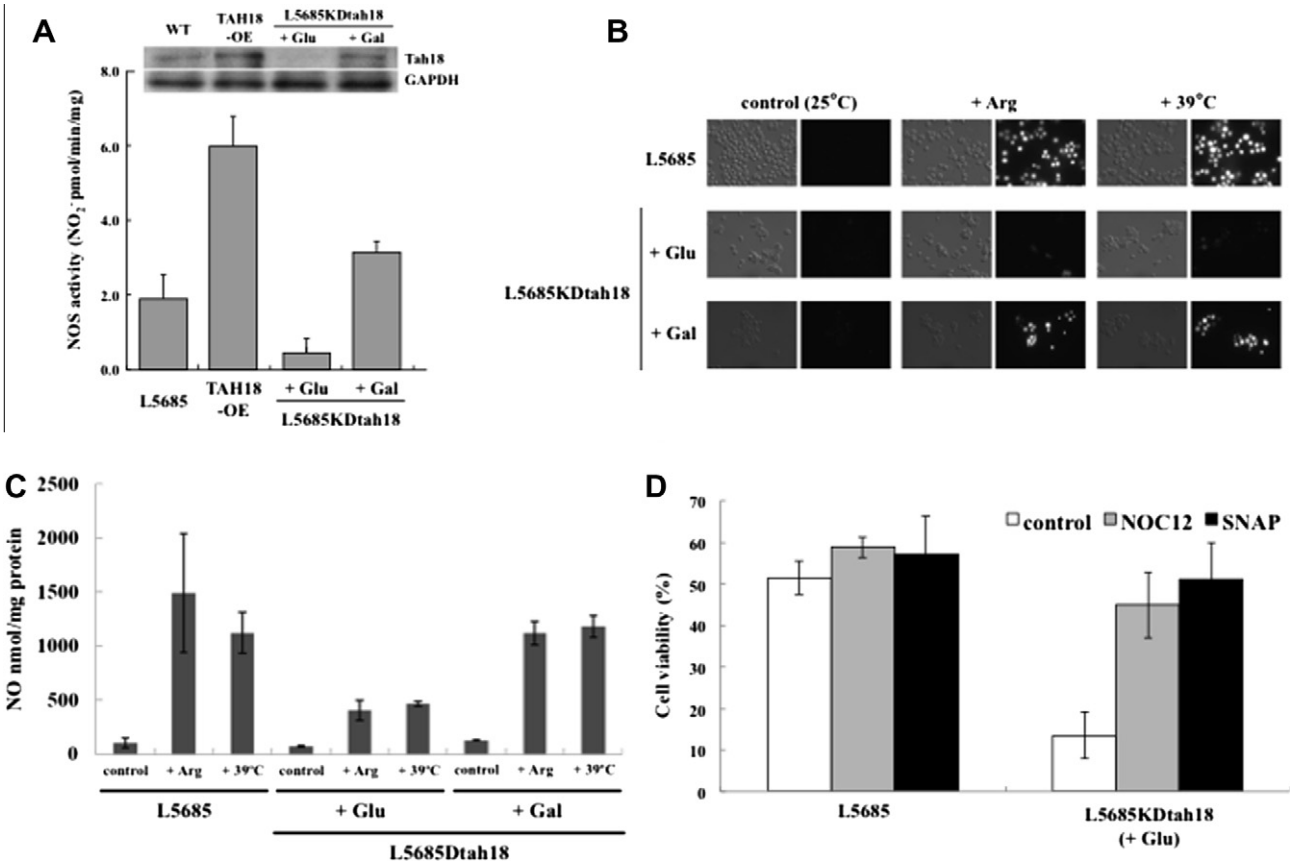

Fig. 4. Tah18-dependent NO production and stress tolerance in yeast cells. (A) NOS activity was measured in L5685, TAH18-OE, and L5685KDtah18 cells. L5685 and TAH18-OE cells were cultured in SD medium, and L5685KDtah18 cells were cultured in SD medium (+Glu) or SG medium (+Gal). Total proteins (10 μg) in the soluble extract were subjected to 12.5% SDS–polyacrylamide gel electorphoresis, and Tah18 and the internal control protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were detected using each antibody. The values are the means and standard deviations of nine independent experiments. (B) NO production was shown as fluorescence from DAF-FM DA in L5685 and L5685KDtah18 cells exposed to 100 μM L-arginine for 1 h (Arg) or 39 °C for 4 h (39 °C) (right panels). Cell morphology was observed through differential interference contrast (left panels). (C) NO production was quantitatively analyzed in L5685 and L5685KDtah18 cells exposed to 100 μM L-arginine for 1 h (Arg) or 39 °C for 4 h (39 °C). An NO standard curve was established by determining the fluorescence of DAF-FM in the presence of various concentrations of NOC7. The values are the means and standard deviations of three independent experiments. (D) Cell viability was tested in L5685 and L5685KDtah18 after exposure to 39 °C for 14 h with or without 40 μM NOC12 or 62.5 μM SNAP. L5685KDtah18 cells were cultured in SD medium (+Glu). The values are the means and standard deviations of nine independent experiments.

the viability of L5685KDtah18 cells grown in glucose-containing medium was significantly lower than that of L5685 cells (Fig. 4D). Treatment with NOC12 or SNAP, on the other hand, restored the survival rate of L5685KDtah18 cells, to a level close to that in L5685 cells (Fig. 4D). Our results showed that Tah18-dependent NO synthesis confers stress tolerance on yeast cells.

Tah18 performs an indispensable role in Fe–S protein biogenesis [26]. To examine whether the stress sensitivity of L5685KDtah18 cells is related to the Fe–S protein biogenesis, we measured the sulfite reductase activity of Met5, which is one of the cytosolic Fe–S proteins (Fig. 3C). The sulfite reductase activity was remarkably reduced in L5685KDtah18 cells. Interestingly, the deficiency in sulfite reductase activity was not restored by the addition of SNAP, indicating that NO production is independent of the Fe–S protein biogenesis.

4. Discussion

Our data provided direct evidence that Tah18-dependent NO synthesis confers high-temperature stress tolerance on yeast cells. NO plays an important role in mammals mainly through the activation of sGC and the posttranslational modification of proteins. Genome-wide analysis has failed to identify sequences that are homologous to mammalian sGC in *S. cerevisiae*. One can postulate the mechanisms by which NO protect yeast cells from oxidative damage. First, NO might provide protective effects by *S*-nitrosylation of the critical L-cysteine residue(s) in the protein from ROS. Whereas ROS irreversibly interact with L-cysteine residues, the NO interaction is reversible [27]. In addition, NO could function as a signaling molecule in the activation of the cellular antioxidant defense system. Human thioredoxin 1 (Trx) is *S*-nitrosylated at position 69, and this *S*-nitrosylation is required for scavenging ROS and for preserving the redox regulatory activity of Trx [28]. NO also regulates the expression of antioxidative genes via *S*-nitrosylation of the transcriptional factor in animals, plants, and bacteria [1,29,30].

Tah18 is a member of the diflavin reductase family, containing FAD and FMN, which are reduced by NADPH. Tah18 could transfer electrons from NADPH to the Fe–S clusters of Dre2 via FAD and FMN [26]. However, Tah18 does not have the oxygenase domain of NOS, which functions as a center of the L-arginine oxidation using two electrons from the reductase domain, in the primary structure (Fig. S3A). Thus, it is important to clarify the oxidizing power and the Dre2's role for full display of Tah18-associated NOS activity. To examine whether Tah18 is the sole NO forming enzyme or is just part of a NOS-like protein, we should confirm enzymatic oxidation of the guanidinium nitrogen on L-arginine into NO using ^{15}N and $^{18}O_2$ with stoichiometric formation of L-citrulline. Random and site-directed mutagenesis experiments are also in progress for identification of crucial residues in Tah18 involved in NO synthesis.

Our data strongly suggest that NO production is independent on nitrite reductase. Nitrite can be chemically reduced to NO under the reducing and acidic conditions, but such conditions were not used in our experiments. Therefore, we believe that NO production in *S. cerevisiae* is dependent on NOS activity. Although Tah18 was suggested to relocalize to the mitochondria after exposure to H_2O_2 [25], Tah18-dependent NO synthesis may occur in the cytoplasm even after exposure to 39 °C, because specific transport of L-arginine into the mitochondria has so far been unknown.

In a search of protein databases, the *S. cerevisiae* Tah18 homologues are widely present in yeasts and fungi (Fig. S3B). Based on the fact the Mpr1 homologues are found only in yeasts and fungi, it appears that such a unique cell protection mechanism involving NO synthesis is highly conserved in yeasts and fungi. Thus, the inhibition of Mpr1 and/or Tah18 activity may be a promising target for the development of antifungal drugs. Yeast is an important unicellular microorganism as a model of higher eukaryotes, because many essential cellular processes are conserved between yeasts and humans. NO is known to be related to many human diseases, such as neuronal disease, hypertension, aging, cancer, etc. Thus, yeast *S. cerevisiae* would be used as a model organism for the therapy in NO-related disease.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.11.023.

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