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Activation of Aro80 transcription factor by heat-induced aromatic amino acid influx in *Saccharomyces cerevisiae*

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ABSTRACT

In *Saccharomyces cerevisiae*, transcription of *ARO9* and *ARO10* genes, involved in the catabolism of aromatic amino acids, is activated by Aro80 transcription factor in response to aromatic amino acids. Here we show that the transcription of *ARO9* and *ARO10* is also induced by heat shock in an Aro80-dependent manner. However, heat shock-related signaling pathways including PKA, PKC, and HOG pathways are not involved in the heat shock activation of Aro80. We elucidate that heat-induced increase in aromatic amino acid influx can lead to the inducer-dependent activation of Aro80 upon heat shock. Known aromatic amino acid permeases play an insignificant role in the heat-induced expression of *ARO9* and *ARO10*, suggesting that an increase in plasma membrane fluidity might be responsible for the influx of aromatic amino acids during heat shock stress.

1. Introduction

In *Saccharomyces cerevisiae*, genes involved in the utilization of various nitrogen sources are regulated by a global nitrogen quality control known as nitrogen catabolite repression (NCR) and by pathway-specific regulators [1]. NCR is mediated by GATA transcription factors consisting of two activators (Gat1 and Gln3) and two repressors (Gzf3 and Dal80) [2]. The NCR-sensitive genes involved in the utilization of alternative nitrogen sources are repressed in the presence of good nitrogen sources such as glutamine and ammonia, and activated in the presence of poor nitrogen sources such as proline and urea [3]. Although it is not completely understood how different nitrogen sources regulate GATA factors, TOR (target of rapamycin) kinase signaling pathway is in part involved in the negative regulation of Gat1 and Gln3 [1,4,5].

Aromatic amino acids are catabolized to the corresponding aromatic alcohols via Ehrlich pathway consisting of three steps; transamination of an amino acid to an α-keto acid, decarboxylation of an α-keto acid to an aldehyde, and reduction of an aldehyde to an alcohol [6–8]. Aro9 transaminase and Aro10 decarboxylase are involved in the first two steps of the aromatic amino acid catabolism via Ehrlich pathway [8,9]. Transcription of the *ARO9* and *ARO10* genes is regulated both by Aro80, a pathway-specific regulator, and by GATA activators [10,11]. Aro80, a Zn_2Cys_6 transcriptional activator, constitutively binds to the promoters of *ARO9* and *ARO10*, and activates transcription in the presence of aromatic amino acids [10]. In addition, we have shown recently that Aro80 is required for the recruitment of Gat1 and Gln3 to the *ARO9* and *ARO10* promoters upon the inhibition of TOR kinase by rapamycin [10].

In this paper, we demonstrated a novel Aro80-depedent activation mechanism of *ARO9* and *ARO10* expression upon heat shock. We showed that heat-induced influx of aromatic amino acids is mainly responsible for the activation of Aro80 upon heat shock stress.

2. Material and methods

2.1. Yeast strains, media, and culture conditions

S. cerevisiae BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) was used as a parent strain in this study. Deletion mutants, *aro80Δ*, *pde2Δ*, *ira2Δ*, *gap1Δ*, *agp1Δ*, *tat2Δ*, *bap2Δ*, *bap3Δ*, *wsc1Δ*, *rom2Δ*, *sho1Δ*, and *ssk1Δ*, derived from BY4741 [12] were obtained from EUROSCARF. *gln3Δgat1Δ* (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 gat1Δ::KanMX6 gln3Δ::His3MX6*) and *gap1Δagp1Δ* (*MATa his3Δ1 leu2Δ0 met15Δ0ura3Δ0 gap1Δ::KanMX6 agp1Δ::LEU2*) strains were generated by PCR-mediated gene targeting method as previously described [13]. Yeast cells were cultured in YPD medium (1% yeast extract, 2% bacto-peptone, and 2% dextrose), SC (synthetic complete) medium (0.67% yeast nitrogen base without amino acids, 2% glucose, and 0.2% amino acid drop out mixture), or SD (synthetic defined) medium (0.67% yeast nitrogen base without

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amino acids, and 2% glucose) supplemented with auxotrophic components (120 μg/ml Leu and 50 μg/ml each of His, Met, and Ura).

2.2. qRT-PCR

Total RNA was isolated from *S. cerevisiae* cells and the relative amount of specific mRNA was detected by quantitative RT-PCR (qRT-PCR) as previously described with some modifications [10]. Reaction mixture containing 1 μl cDNA, 1X SYBR master mix (Roche Diagnostics, Germany), and gene-specific primers was subjected to qPCR reaction with 40 cycles of 95 °C for 10 s, 55 °C for 20 s, and 72 °C for 20 s using Roche LightCycler 480 real-time PCR system (Roche Diagnostics, Germany). mRNA levels were analysed by qRT-PCR normalized with *ACT1*. Each value represents the average ± standard deviation from three independent experiments. The specific primers for qPCR of *ARO9*, *ARO10*, *HSP26*, *CTT1*, and *ACT1* were as follows: *ARO9*, 5′-GGAATTTCGATAGACCTGACGA-3′ and 5′-GGAAGCTTCAATCAACTGATC-3′; *ARO10*, 5′-ACTCAATATACG AACGAAACA-3′ and 5′-CTCCTCGAATTCTTAAGTTTG-3′; *HSP26*, 5′-G GTCAAGGTCAAGGAGAGCAG-3′ and 5′-GTGGTTCTTACCATCCTTCT G-3′; *CTT1*, 5′-CAAAGGGATAGTTCTTGACG-3′ and 5′-GACCAAGTCTT GGCATAACC-3′; *ACT1*, 5′-CTGCCGGTATTGACCAAAC-3′ and 5′-GGTG AACGATAGATGGACC-3′.

2.3. Determination of the amino acid concentration by using UPLC/QQQ-MS

To detect intracellular metabolite, samples were prepared as previously described with some modifications [14–16] and analyzed by UPLC/triple quadrupole mass spectrometry (QQQ-MS). Liquid chromatography was performed on a Accela 1250 UPLC™ system (ThermoFisher scientific, USA) with Thermo Fisher Hypersil Gold Hilic column (2.1 × 100 mm, 1.8 μm, ThermoFisher scientific, USA). The temperatures of column oven and auto-sampler were maintained at 30 and 10 °C, respectively. The mobile phases were (A) 5% (vol/vol) acetonitrile, 20 mM ammonium hydroxide, and 20 mM ammonium acetate in water (pH = 9.0), and (B) acetonitrile. The linear gradient program began with 15% (A) for 10 min and proceeded to 98% (A) over 28 min and then returned to initial conditions, 15% (A), which were maintained for 7 min. The total analysis time was 35 min with a flow rate of 400 μl/min and an injection volume of 10 μl using partial-loop mode. Quantification of amino acid was performed using a TSQ quantum Access Max-QQQ mass spectrometer (ThermoFisher scientific, USA). Ionization was performed in the positive and negative switching and heated-electrospray (HESI) mode. Scan time for SRM transition (dwell time) was 5 ms, each discharge current value was 4 μA between positive and negative polarity. The conditions used for the ESI source were as follows: capillary voltage, 4.0 kV; vaporizer temperature, 40 °C; source temperature, 270 °C; and desolvation temperature, 350 °C. Nitrogen was used as the sheath gas with a pressure of 30 psi. The collision energy was set at 23 eV. ThermoFisher Xcaliver interface was used to control mass spectrometry. Table 1 shows the component-dependent SRM setting with retention time for QQQ mass spectrometry.

3. Results and discussion

3.1. Aro80 is activated by heat shock

Transcription of the *ARO9* and *ARO10* genes are regulated by aromatic amino acids and the quality of nitrogen sources [11]. We investigated whether other environmental conditions can affect the expression of these genes. We examined the expression of *ARO9* and *ARO10* under heat shock, osmotic stress, and glucose starvation conditions. Among these stress conditions, only heat shock could activate *ARO9* and *ARO10* (Fig. 1, data not shown). Heat shock induction of *ARO9* and *ARO10* in rich YPD medium was completely abolished in *aro80Δ* strain, suggesting that Aro80 can be activated by heat shock (Fig. 1A). Heat shock induction of *HSP26*, a target of Msn2/4 and Hsf1 transcription factors [17], was not affected by the lack of *ARO80*, confirming the specific role for Aro80 in the heat shock induction of its target genes (Fig. 1A). Gln3 and Gat1 directly activate the expression of *ARO9* and *ARO10* in the presence of poor nitrogen sources or rapamycin [10]. However, heat shock-dependent induction levels of *ARO9* was not affected in a double deletion mutant *gln3Δgat1Δ*, indicating that Gln3 and Gat1 are not involved in the regulation of Aro80 target genes upon heat shock stress (Fig. 1B).

3.2. PKA, PKC, and HOG signaling pathways do not affect the heat shock induction of Aro80 target genes

Protein kinase A (PKA), protein kinase C (PKC), and high osmolarity glycerol (HOG) signaling pathways are implicated in heat shock response in *S. cerevisiae*. Therefore, we tested whether these pathways can regulate Aro80 activation upon heat shock. PKA activity is up-regulated in *pde2Δ* and *ira2Δ* through an increase in cAMP levels and a decrease in GTP hydrolysis of Ras proteins, respectively [18,19]. Heat shock induction of *CTT1*, a known target of Msn2 and Msn4 which are negatively regulated by PKA [20], was dramatically reduced in *pde2Δ* and *ira2Δ* mutants (Fig. 1C). However, expression of *ARO9* was not much affected by the deletion of *PDE2* or *IRA2*, suggesting that PKA pathway might not regulate Aro80 activation. We also examined the possible involvement of HOG signaling pathway in the heat shock induction of *ARO9*. However, deletion mutants of *SHO1* and *SSK1* involved in the HOG signaling pathway [21] showed no defect in the heat shock induction of *ARO9* (data not shown). Furthermore, deletion of *WSC1* and *ROM2* in PKC signaling pathway [22] also showed normal induction of *ARO9* upon heat shock (data not shown).

3.3. Effect of extracellular amino acids on the heat shock induction of ARO9

Since the heat shock induction of *ARO9* in YPD medium was not affected by the known signaling pathways we tested, we hypothesized that the heat shock activation of Aro80 might involve an increase in the availability of aromatic amino acids which act as inducers. The intracellular amino acid levels can be increased either by amino acid uptake from the medium or by internal

Table 1
Component-dependent SRM setting for triple quadrupole mass spectrometry.

Amino acid	Q1[a]	Q3[b]	Collision energy (*V*)	Retention time (min)	Charge
Phenylalanine	166.2	120.16	12	2.80	+
Tyrosine	182.201	136.15	12	3.38	+
Tryptophan	205.2	146.12	16	2.59	+

[a] Parent ion mass.
[b] Daughter ion mass.

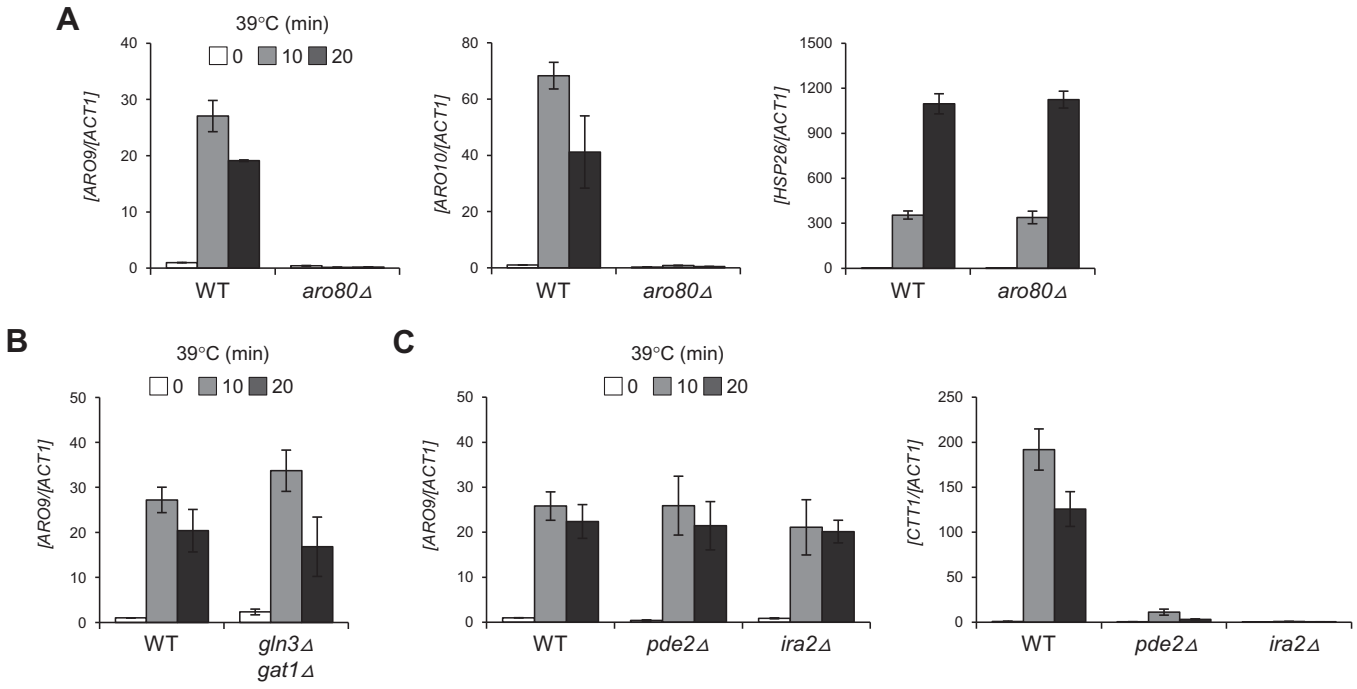

Fig. 1. Expression of *ARO9* and *ARO10* is activated by heat shock in an Aro80-dependent manner. (A) Aro80-dependent induction of *ARO9*, *ARO10* upon heat shock. BY4741 wild type (WT) and *aro80Δ* cells, grown in YPD medium at 30 °C, were heat shocked at 39 °C for 10 or 20 min. mRNA expression levels of *ARO9*, *ARO10*, and *HSP26* were analyzed by qRT-PCR normalized with *ACT1*. Each value represents the average of three independent experiments, and error bars indicate standard deviations. (B) Effect of Gat1 and Gln3 on the heat shock induction of *ARO9*. *ARO9* mRNA expression levels were analyzed by qRT-PCR in WT and *gln3Δgat1Δ* before and after heat shock. (C) Effect of PKA pathway on the heat shock induction of *ARO9*. *ARO9* and *CTT1* mRNA levels were analyzed by qRT-PCR in WT, *pde2Δ*, and *ira2Δ* before and after heat shock.

supply of free amino acids through protein degradation, autophage, or biosynthesis. To elucidate the regulatory mechanism, we examined whether *ARO9* and *ARO10* could be induced by heat shock in the absence of aromatic amino acids in the medium. In a minimal medium lacking amino acids (−AA) except for auxotrophic supplements (His, Leu, and Met), both basal and heat-induced expression levels of *ARO9* and *ARO10* were lower than those in a minimal medium containing all amino acids (+AA) (Fig. 2). *ARO9* and *ARO10* were induced only by 3.6- and 1.8-fold, respectively, in the medium lacking aromatic amino acids, but by 7.6- and 10.1-fold in the presence extracellular amino acids. Therefore, an increase in amino acid uptake rather than internal supply of free amino acids might be mainly responsible for the Aro80 activation upon heat shock.

3.4. Heat shock induces an increase in intracellular concentrations of aromatic amino acids

Next, we investigated the changes in the intracellular concentrations of aromatic amino acids upon heat shock depending on the medium types. Cells were grown in the presence or absence of amino acids at 30 °C, and then heat shocked at 39 °C for 15 min. The intracellular amino acid levels were detected by mass spectrometry-based analysis, UPLC/QQQ-MS. When cells were grown in a medium containing amino acids (+AA), intracellular Phe, Tyr, and Trp concentrations increased by 1.5-fold in average upon heat shock (Fig. 3). On the other hand, in cells grown in the absence of amino acids (−AA), the concentrations of Phe and Tyr were not significantly changed before and after heat shock, and

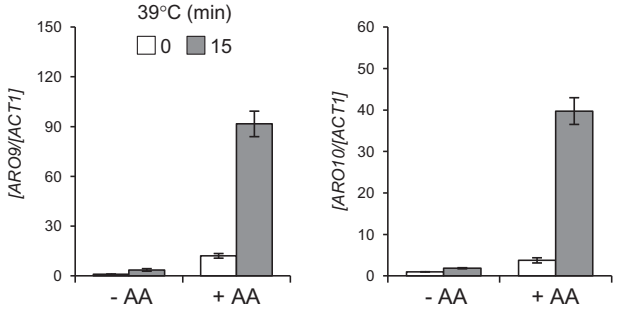

Fig. 2. Effect of extracellular amino acids on the heat shock induction of *ARO9* and *ARO10*. Wild type cells were grown in a minimal medium lacking amino acids (−AA) or containing amino acids (+AA) until early exponential phase and then heat shocked at 39 °C for 15 min. *ARO9* and *ARO10* mRNA levels were analyzed by qRT-PCR normalized with *ACT1*.

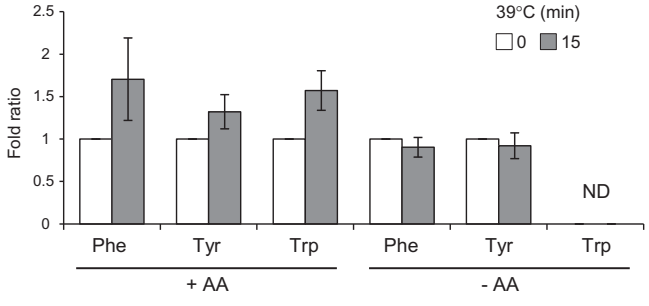

Fig. 3. Increase in intracellular aromatic amino acid concentrations upon heat shock. BY4741 cells were grown in a minimal medium lacking amino acids (−AA) or containing amino acid (+AA) until early exponential phase and then heat shocked at 39 °C for 15 min. Intracellular metabolites were analyzed by UPLC/QQQ-MS. Results are presented as fold-increases in Phe, Tyr, and Trp concentrations upon heat shock compared with the untreated controls. ND indicates not detectable. The mean ± standard deviation of three independent experiments is shown.

Trp concentration was below the detection limit. These results support our previous hypothesis that heat shock might induce an increase in amino acids uptake from outside of the cells.

3.5. Amino acid permeases do not affect the heat shock induction of *ARO9* and *ARO10*

The heat-induced amino acid uptake could be mediated by plasma membrane amino acid permeases. On the other hand, it could be a result of heat-induced increase in membrane fluidity [23,24], which might facilitate passive transport of amino acids along with other molecules. To elucidate the mechanism, we investigated the roles of plasma membrane amino acid permeases in the heat-induced expression of *ARO9* and *ARO10*. Gap1, Agp1, Tat2, Bap2, and Bap3 permeases are known to be involved in the transport of aromatic amino acids [25]. However, heat-induced expression levels of *ARO9* were not significantly changed in the deletion mutants lacking each permease (Fig. 4A).

It has been known that Aro80 is also indirectly activated by an increase in intracellular aromatic amino acids in the presence of poor nitrogen sources or rapamycin [10,11]. In this case, GATA factor-dependent transcriptional induction and stabilization of amino acid transporters are responsible for the increase in aromatic amino acid uptake [10,11]. However, a double deletion mutant *gap1-Δagp1Δ*, which has shown a defect in the Trp-dependent activation of *ARO9-lacZ* in a urea medium [11], showed normal induction of *ARO9* and*ARO10* by heat shock. These results suggest that amino acid permeases might not play a major role in the heat-induced amino acid uptake. Although we cannot rule of the possibility that other yet uncharacterized amino acid permeases are responsible for the aromatic amino acid uptake during heat shock, increase in membrane fluidity might be the key reason for the heat-induced amino acid influx, which in turn activates Aro80, leading to the induction of *ARO9* and *ARO10* genes.

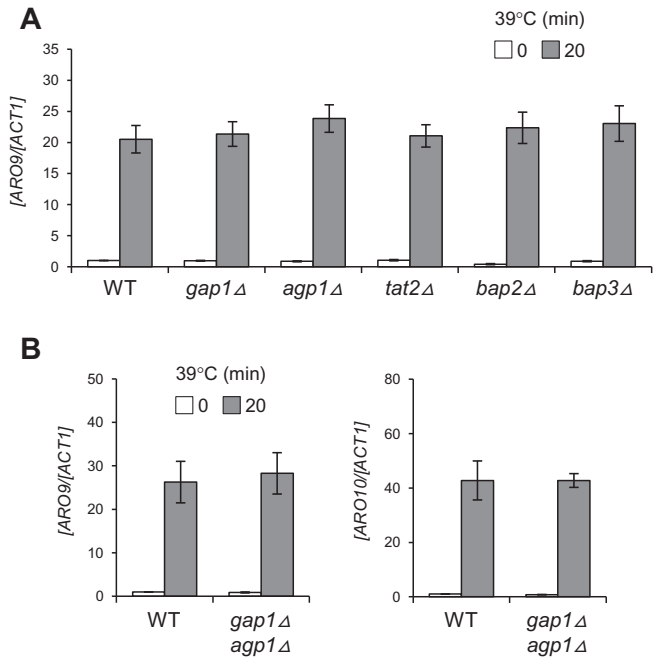

Fig. 4. Effect of amino acid permeases on the heat shock induction of *ARO9* and *ARO10*. (A) WT, *gap1Δ*, *agp1Δ*, *tat2Δ*, *bap2Δ*, and *bap3Δ* cells grown in YPD medium were heat shocked at 39 °C for 20 min, and *ARO9* mRNA levels were analyzed by qRT-PCR. (B) Effect of *agp1Δgap1Δ* on the heat shock induction of *ARO9* and *ARO10*. Heat shock induction of *ARO9* and *ARO10* mRNA was analyzed by qRT-PCR in WT and *agp1Δgap1Δ*.

In summary, our study suggests a novel regulatory pathway for the heat-induced gene expression. In addition to regulating classical signal transduction pathways such as PKA and PKC, and activating heat shock transcription factor (Hsf1), heat shock might affect gene expression via changing intracellular concentrations of various metabolites, some of which can function as activators or repressors of various transcription factors. Heat shock might affect the profile of cellular metabolites by modulating membrane permeability as well as by regulating various metabolic pathways, which are also influenced by nutrients and other environmental conditions. Comprehensive analysis of the heat-induced changes in metabolite concentrations combined with the global gene expression data might provide more clear insight into the cellular responses to heat shock.

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