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Transcriptional regulation of *ARO9* and *ARO10* genes for the catabolism of aromatic amino acids in *Saccharomyces cerevisiae*

***Saccharomyces cerevisiae*의 방향족 아미노산 이화과정에 관여하는 *ARO9*과 *ARO10* 유전자의 전사 조절 기작**

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이 규 성

Transcriptional regulation of *ARO9* and *ARO10* genes for the catabolism of aromatic amino acids in *Saccharomyces cerevisiae*

By

Kyusung Lee

Advisor: Professor Ji-Sook Hahn, Ph.D.

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School of Chemical and Biological Engineering
Graduate School
Seoul National University

Abstract

Transcriptional regulation of *ARO9* and *ARO10* genes for the catabolism of aromatic amino acids in *Saccharomyces cerevisiae*

Kyusung Lee

School of Chemical and Biological Engineering

The Graduate School

Seoul National University

Saccharomyces cerevisiae can utilize aromatic amino acids as the sole nitrogen source. The final products of aromatic amino acid catabolism are aromatic alcohols, including tryptophol, phenylethanol, and tyrosol. Production of aromatic alcohols from aromatic amino acids is known to proceed via the Ehrlich pathway. It consists of three main 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. *ARO9* and *ARO10* genes encode transaminase and decarboxylase, respectively, in the Ehrlich pathway.

In this thesis, the activation mechanisms of *ARO9* and *ARO10* genes via Aro80, a pathway-specific transcriptional activator, and GATA factors, global nitrogen regulators, were elucidated.

Firstly, inducer-dependent activation mechanism of Aro80 was investigated. It was found that Aro80 constitutively localized in the nucleus irrespective of the availability of inducers such as aromatic amino acids, methionine, or aromatic alcohols. In addition, it was demonstrated that Aro80 is constitutively bound to its target promoters and is activated by inducers at the level of transactivation. Although Aro80 was also shown to bind to its own promoter, *ARO80* expression was not induced by tryptophan.

Secondly, direct involvement of GATA factors in *ARO9*, *ARO10* and *ARO80* was demonstrated. It was shown that Aro80 is absolutely required for Gat1 binding to the *ARO9*, *ARO10,* and *ARO80* promoters upon TORC1 inhibition by rapamycin treatment. Gln3 binding to these promoters showed a partial requirement for Aro80. Rapamycin-dependent Gat1 and Gln3 binding to the Aro80 target promoters was not affected by tryptophan availability, suggesting that transactivation activity of Aro80 is not necessary for the recruitment of GATA factors. Rapamycin-dependent induction of Aro80 target genes also required PP2A phosphatase complex, but not Sit4 phosphatase, acting downstream of TORC1

Thirdly, it was shown 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 were not involved in the heat shock activation of Aro80. It was elucidated 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.

Fourthly, a series of novel inducible promoters were generated based on the *ARO9* promoter. The promoters with various promoter strengths could be generated by modulating the number of Aro80 binding site combined with either *ARO9* or *ARO80* core promoter. Expression levels from these promoters could be further regulated by tryptophan concentration, suggesting potential application of these inducible promoters for fine tuning of gene expression levels in metabolic engineering.

Keywords: Aromatic amino acid catabolism, nitrogen catabolite repression, Aro80, GATA factor, heat shock and promoter engineering

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List of Abbreviations

AA : Amino acid

cAMP : Cyclic adenosine-monophosphate

ChIP : Chromatin immunoprecipitation

DAPI : 4,6 diamidino-2-phenylindol

DBD : DNA-binding domain

EDTA : Ethylenediaminetetraacetic acid

EGFP : Enhanced green fluoresence protein

EMSA : Electrophoretic mobility shift assay

FKBP12 : FK506 binding protein

GC : Gas chromatography

GST : Gluthathione-s-transferase

HOG : High osmolarity glycerol

IB : Immuno blot

IN : Input

IP : Immunoprecipitation

NCR : Nitrogen catabolite repression

PAGE : Polyacrylamide gel electrophoresis

PIKK : Phosphatidyl inositol kinase-related kinase

PKA : Protein kinase A

PKC : Protein kinase C

PP2A : Protein phosphatase 2A

QQQ-MS: Triple quadrupole mass spectrometer

qRT-PCR : Quantitative reverse transcription polymerization chained reaction

RT-PCR : Reverse transcription polymerization chained reaction

SDS : Sodium dodecyl sulfate

SLAD : Synthetic low ammonia dextrose

STRE : Stress response element

TAP : Tandem affinity purification

TAP42 : Type 2A phosphatase-associated protein 42

TCA : Trichloroacetic acid

TORC1/2 : Target of rapamycin complex 1/2

UAS : Upstream activation sequence

UPLC: Ultra performance liquid chromatography

Chapter 1.

Research background and objective

1.1. Research background

Saccharomyces cerevisiae can utilize aromatic amino acids as the sole nitrogen source [1,2]. The final products of aromatic amino acid catabolism are aromatic alcohols, tryptophol, phenylethanol, and tyrosol, respectively [3,4]. Aromatic alcohol production from aromatic amino acids is known to process via the Ehrlich pathway [5]. It consists of three main 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. *ARO9* and *ARO10* genes encode transaminase and decarboxylase, respectively, in the Ehrlich pathway [6,7]. Tryptophan is converted to tryptophol via three enzymatic steps, where phenylalanine and tyrosine are transformed to phenylethanol and tyrosol in a similar reaction. Among these aromatic alcohols, tryptophol and phenylethanol act as quorum sensing molecules in *S. cerevisiae* [4,8].

The transcription of *ARO9* gene is induced in the presence of tryptophan, phenylalanine, or tyrosine in the growth medium and the level is very low in the absence of aromatic amino acids [2,9]. The expression of *ARO9* is also modulated by nitrogen quality via an ammonia effect [2]. Therefore *ARO9* gene appears to be regulated in two regulatory pathways by inducers and nitrogen sources, like a well-known nitrogen catabolite repression (NCR) genes. The promoter of *ARO10* gene contains a similar sequence of *ARO9* promoter [10]. This gene is also subjected to aromatic amino acid-dependent induction and regulated by nitrogen quality.

Previously, research on *ARO9* and *ARO10* genes has focused towards the understanding of the production of aromatic alcohols as quorum sensing molecules in *S. cerevisiae*, and the induction of *ARO9* and *ARO10* genes via Aro80 and nitrogen

quality was characterized [2,4,9,11]. However, regulatory mechanism involved in their expression has not been well understood

Aro80 is known as a member of the Zn_2Cys_6 transcription factor [12,13]. The amino acid sequence of Aro80 protein contains several motifs that characterize the Gal4 family of transcription factors. Tryptophan dependent induction of both *ARO9* and *ARO10* genes is abolished in *ARO80* deletion mutant, which indicates the important role of Aro80 for the expression of *ARO9* and *ARO10* under tryptophan treatment [2]. However, too little is known about tryptophan-dependent activation mechanism of transcription activator Aro80 involved in aromatic amino acid utilization.

When good nitrogen sources like glutamine, asparagine or ammonia are supplemented to the growth medium of *S. cerevisiae* growing in a poor nitrogen source like proline or urea, the transcriptional expression of some enzyme and permease genes are involved in the utilization of the poorer nitrogen source is repressed, and their corresponding products are inactivated. It is well-known as nitrogen catabolite repression (NCR) [1,14]. The transcription of the NCR genes is regulated by the GATA transcription factors Gln3 and Gat1 [15,16]. The binding sites of GATA factors in NCR-sensitive promoters contain the core GATAA sequence that binds to the well characterized zinc finger motif [1,17]. The regulation mechanism of GATA factors is exerted at the level of cellular localization, which in some cases correlates with their phosphorylation status via TORC1 signaling pathway[16,18]. In previous studies, *ARO9* and *ARO10* were found to be under the control of nitrogen catabolite repression [2,19], but the direct roles for GATA factors, Gat1 and Gln3, in this regulation have not yet been elucidated.

Heat shock physiologically affects various cellular responses including metabolic reprogramming, alteration in cell wall, membrane dynamic, protein aggregation, and sequestration [20,21,22]. In addition to the physiological changes, yeast cells respond to heat shock by dramatically altering their gene expression programs. Interestingly, many of the induced genes by heat shock are not required for heat shock survival. A possible elucidation is provided by the observation that the induction of genes by heat shock is required not for survival of the stimulating stress but rather for survival of a succeeding stress. Also it is well-known that most of the induced genes by heat shock are regulated by heat shock factor (Hsf1) and Msn2/4. Sometime Hsf1 and Msn2/4 co-regulate their target genes [21]. However, too little is known about the heat shock-dependent activation mechanism of aromatic amino acid utilization genes.

Promoter engineering is an important part of metabolic engineering [23,24]. In yeasts, there are now many promoter systems available that allow one to increase the expression of genes by more than 1000-fold and activate gene expression at a particular time point during a growth phase. Because expression levels of these promoters are extremely low or high, many researches have recently focused on the optimization of promoter strengths via promoter engineering [23,25,26]. There are many factors that influence the promoter strengths in *S. cerevisiae;* including the sequence context on regulatory motifs, the location of transcription factor binding sites, the number of transcription factor binding sites and nucleosome-disfavoring sequences [27,28,29,30,31,32]. Therefore, these factors are not only dispensable but, when combined together, they are sufficient for developing promoter strengths. Recently, hybrid promoter concepts have been conducted to control the promoter strengths in *S. cerevisiae*[33]. Synthetic hybrid promoters consist of a core promoter region fused to a

single upstream activating sequence or tandem upstream activation sequence repeats that modulate the promoter strength[34]. These upstream activation sequences help localize specific transcription factors that increase the transcriptional activity of the core promoter[24]. Therefore the upstream activation sequence (UAS) and core promoter regions serve as modular synthetic parts that can be combined to produce a strong, functional UAS-core promoter module. For example, it was shown that putative Gal4 binding sites were fused individually or in combination with the P_{LEUM} core promoter, generating a library of hybrid promoters differing in expression when induced by galactose treatment [33].

1.2. The objective of this study

S. cerevisiae can utilize aromatic amino acids as the sole nitrogen source. The catabolism of aromatic amino acids is known to process via the Ehrlich pathway. The degradation of aromatic amino acids consists of three main 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. *ARO9* and *ARO10* genes encode transaminase and decarboxylase, respectively, in the Ehrlich pathway. Therefore the expression of *ARO9* and *ARO10* genes are important for cell metabolism. However, the regulatory mechanism involved in their expression has not been well understood. In previous studies, *ARO9* and *ARO10* were known to be under the control of nitrogen catabolite repression, but the direct roles for GATA factors, Gat1 and Gln3, in this regulation have not yet been elucidated. Heat shock physiologically affects various cellular responses including metabolic reprogramming, alteration in cell wall, membrane

dynamic, protein aggregation, and sequestration. In addition to the physiological changes, yeast cells respond to heat shock by dramatically altering their gene expression programs. However, too little is known about heat shock-dependent activation mechanism of transcription activator Aro80 involved in aromatic amino acid utilization.

This thesis focuses on the identification of regulatory mechanism of *ARO9* and *ARO10* genes in response to various ligands and environmental conditions in *S. cerevisiae*. Novel activation factors and their roles were characterized, and regulatory mechanisms were studied. In addition, using characterized *ARO9* promoter, biotechnological application was applied.

Chapter 2.

Literature review

2.1. Amino acids catabolism in *S. cerevisiae*

2.1.1. Eukaryotic transcription factors as direct nutrient sensors

The zinc finger proteins form one of the largest families of transcriptional regulators in eukaryotes. Most of zinc finger proteins interact with target promoters, thereby playing crucial roles in transcription and translation. Also zinc finger proteins are involved in various cellular processes, including chromatin remodeling, lipid binding, protein stabilizing and zinc sensing [12,13].

The structure of zinc cluster proteins can be divided into three functional domains: the DNA binding domain (DBD), the regulatory domain, and the activation domain (Fig 2.1 A). Also, the DNA binding domain consists of sub regions: the zinc finger region, the linker region, and the dimerization region. It is well-known that these regions are required for protein-DNA and protein-protein interactions and specificity for DNA-binding [13]. Furthermore the activity of zinc cluster proteins is regulated by nuclear-cytoplasmic shuffling, the affinity of DNA binding, phosphorylation, de-phosphorylation and unmasking of the activation domain. Some of zinc cluster transcriptional regulators are important for the expression of amino acid metabolism genes in *S. cerevisiae* (Fig 2.1 B) [13]. For example, Lue3 is an activator or repressor of branched amino acid biosynthesis genes [35,36]. And Cha4 regulates the transcription of enzyme genes for catabolism of threonine [37]. Also Lys14 is required for expression of lysine synthesis genes [38].

Especially, Zinc finger transcriptional activator Aro80 positively regulates the transcription of *ARO9* and *ARO10* genes involved in catabolism of aromatic amino

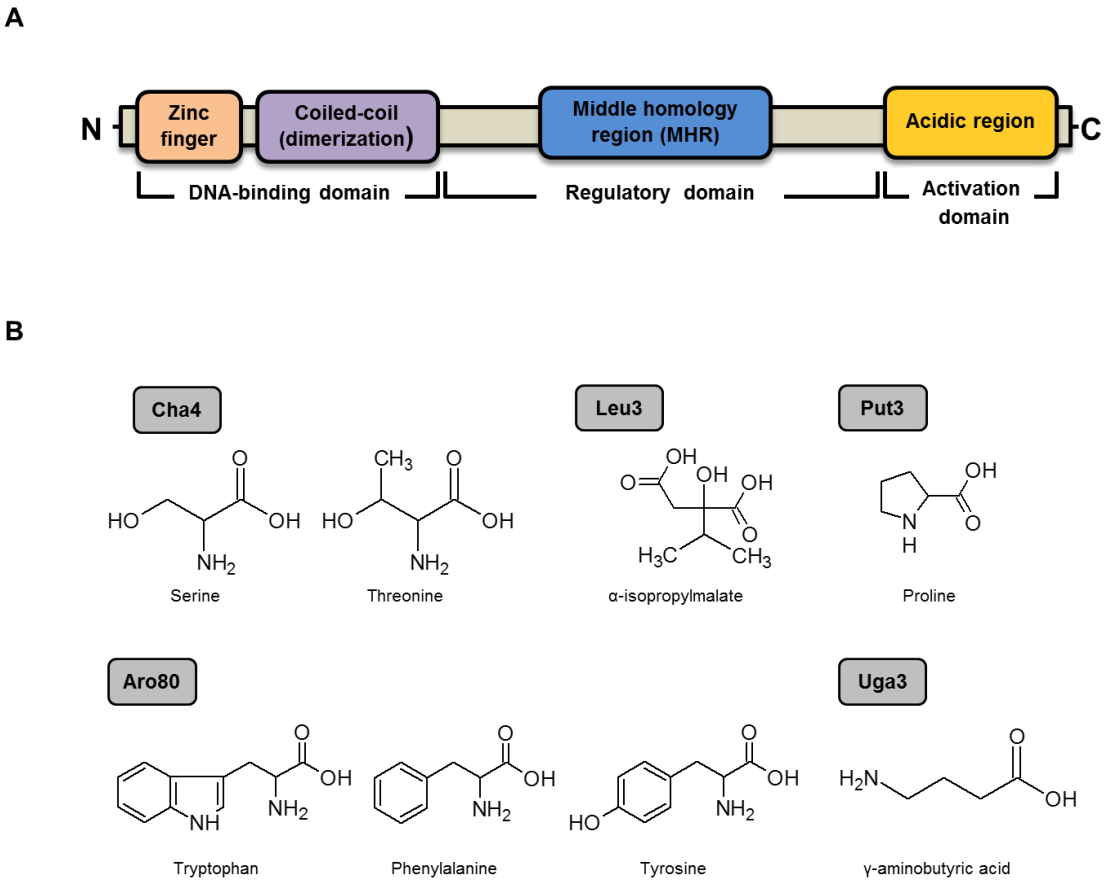


Figure 2.1 Eukaryotic transcription factors as direct nutrient sensors.

A. Functional domains of zinc cluster proteins. Zinc cluster proteins can be separated into three functional domains: the DBD, the regulatory domain, and the acidic region

B. Eukaryotic transcription factors as direct nutrient sensors

acids [2]. *ARO9* gene encodes an aromatic aminotransferase II involved in the first catabolic step of tryptophan, phenylalanine and tyrosine [39]. *ARO10* gene encodes a decarboxylase [7]. The transcription of *ARO9* and *ARO10* is induced in the presence of aromatic acids and in the presence of a poor nitrogen source such as urea and proline, which is repressed in the presence of a good nitrogen source such as glutamine or ammonia [2]. Some branched chain amino acids induce the expression of *ARO9* and *ARO10* via Ehrlich Pathway in *S. cerevisiae* [9].

Ehrlich pathway is a well-known pathway for amino acid catabolism in *S. cerevisiae* (Fig 2.2). In Ehrlich pathway, following transamination of an amino acid into the corresponding 2-keto acid, the 2-keto acid decarboxylates to an aldehyde [5,40]. And then the aldehydes can be either oxidized by aldehyde dehydrogenases to organic acids or reduced by alcohol dehydrogenases to alcohols, which are called fusel alcohols.

Especially, *S. cerevisiae* can utilize aromatic amino acids as a sole nitrogen source via Ehrlich pathway, with the main products of their catabolism being tryptophol, 2-phenethyl alcohol or tyrosol, respectively, which are collectively known as fusel alcohols [5]. Aromatic amino acids directly activates transcriptional factor Aro80, where Aro80 is required for expression of *ARO8* and *ARO9* (encodes aromatic transaminases) and *ARO10* (encodes aromatic decarboxylase). Yeast cells cannot use the tryptophan as a sole nitrogen source by deletion of *ARO80* gene [2]. It means that aromatic amino acid utilization by Ehrlich pathway requires the expression of *ARO9* and *ARO10* via transcription factor Aro80.

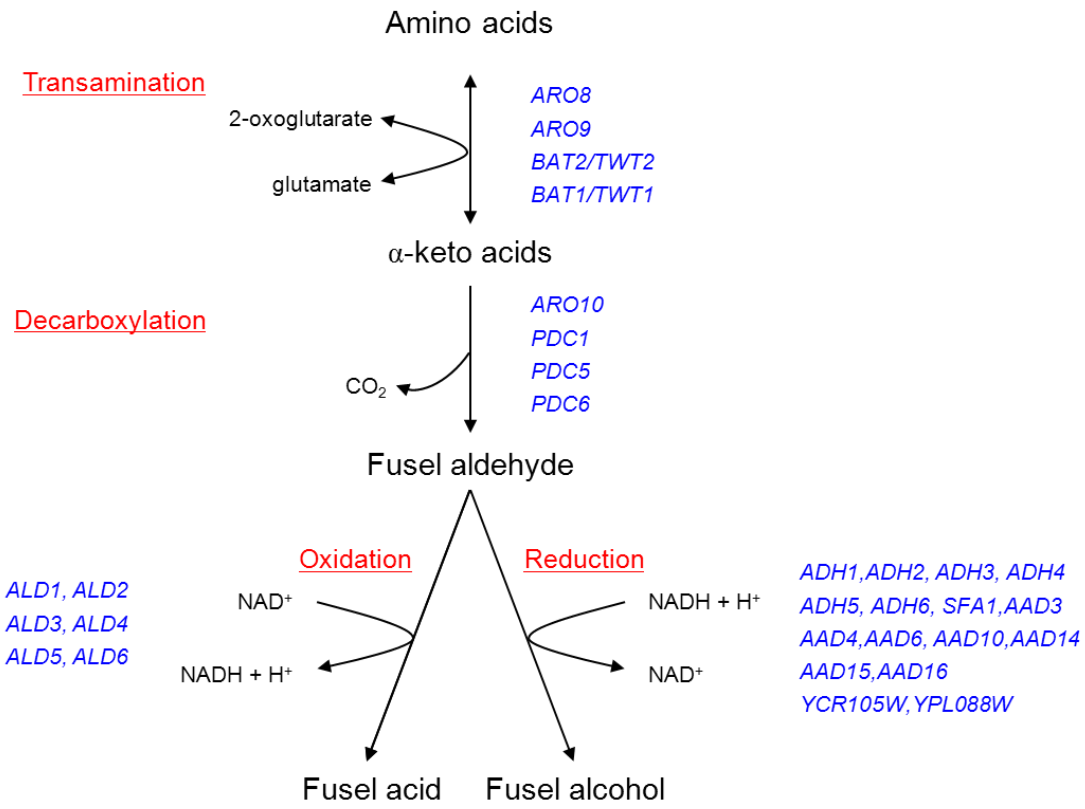


Figure 2.2 The Ehrlich pathway in *S. cerevisiae.*

Catabolism of branched-chain amino acids, aromatic amino and the sulfur-containing amino acid leads to the formation of fusel acids and fusel alcohols in *S. cerevisiae*. The genes encoding the enzymes of each step are indicated

2.2. Target of Rapamycin (TOR) signaling pathway

2.2.1. TOR signaling pathway

S. cerevisiae senses and responds to the environment and amount of available nutrients [41]. The target of rapamycin (TOR) kinases belongs to a family of protein kinase termed the phosphatidyl inositol kinase-related kinase (PIKK) and functions as a key regulator of eukaryotic cell growth in response to nutrient availability [42]. Many components in the TOR complexes are highly conserved in yeasts to mammals [41]. The target of rapamycin proteins in *S. cerevisiae* consists of TOR complex1 and TOR complex2. TORC1 and TORC2 show a central role in controlling cell growth and cell proliferation as part of two distinct signaling branches [41]. Although structurally similar, TOR1 and TOR2 are functionally distinguishable. In *S. cerevisiae*, TOR1 associates with the conserved proteins Tco89, Kog1 and Lst8 to form TORC1 and modulates cell proliferation and the transition between growth and quiescence [41].

TOR2 is found generally as a component of TORC2, but it can directly bind to TORC1 components under deletion of TOR1. TORC2 consists of Avo1, Avo2, Avo3, Bit61, Lst8 and TOR2 and regulates the cell polarity and the organization of the actin cytoskeletons [41].

Rapamycin acts and inhibits cell growth by restricting the function of TORC1 kinase activity (Fig 2.3) [43,44]. Rapamycin forms a complex with yeast FKBP12 homologue, Fpr1, and this rapamycin-Fpr1 complex directly inhibits TORC1 activity [44]. Rapamycin-sensitive TORC1 signaling pathway plays important roles in cell growth by regulating translation, ribosome biosynthesis, autophagy and transcription of stress

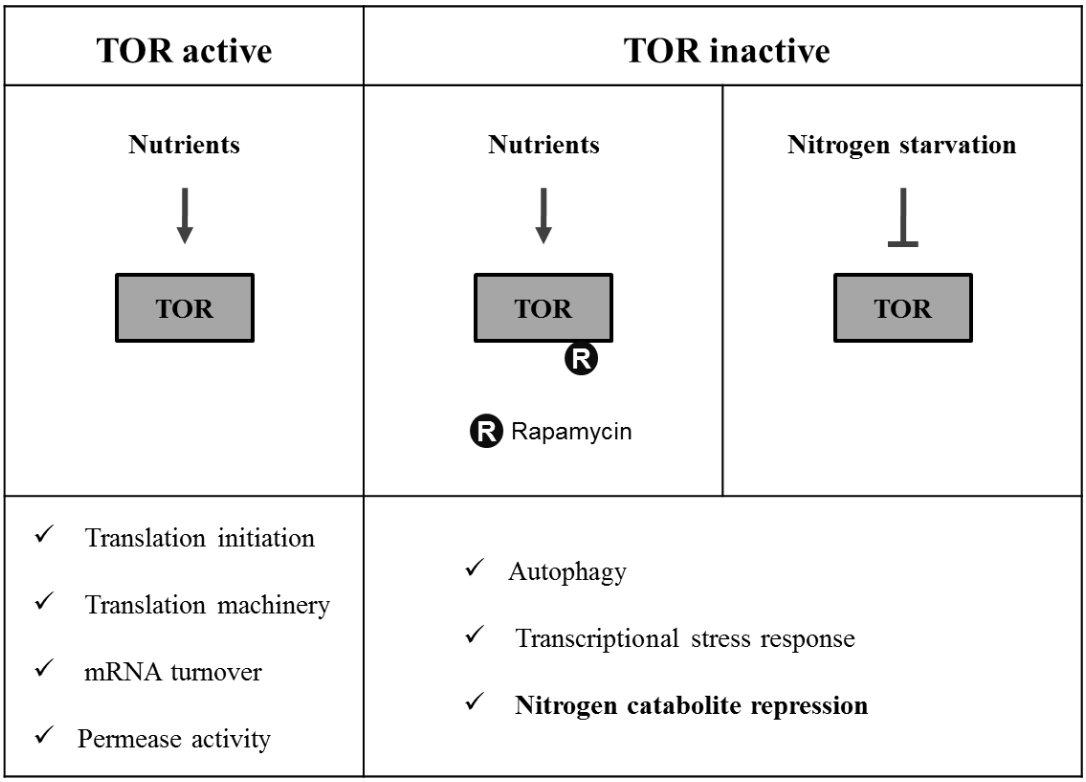


Figure 2.3 Target of rapamycin signaling in *S. cerevisiae*.

related genes (Fig 2.3) [41]. Also, inactivation of TORC1 by rapamycin treatment mimics the nitrogen starvation condition, leading to the induction of transcription of nitrogen catabolite repression (NCR) genes [44].

2.2.2. Regulation of nitrogen catabolite repression (NCR) by GATA factors

S. cerevisiae can utilize alternative or poor nitrogen sources such as proline, urea, allantoin, GABA (γ-aminobutyrate) and arginine when preferred, and good nitrogen sources like glutamine, ammonium and asparagine are unavailable in the environment [1,44]. In the presence of good nitrogen, transcription of genes encoding the proteins needed to transport and degrade poor nitrogen sources does not get induced. On the other hand, the absence of good nitrogen sources results in relief of nitrogen repression at the transcriptional level of the poor nitrogen catabolite pathways. This phenomenon is referred to as nitrogen catabolite repression (NCR) [1]. Regulation of nitrogen catabolite repression gene expression in *S. cerevisiae* is regulated by two positive GATA factors; Gln3 and Gat1 and two negative GATA factors; Gzf3 (Deh1) and Dal80 (Uga43). The central GATAA motif is recognized by four GATA factors to which they bind and exert their functions [15,17,45]. In the good nitrogen sources, Gln3 is inhibited by their Ure2-dependent sequestration in the cytoplasm (Fig 2.4) [15]. As a result, the transcription of NCR genes does not get induced. On the other hand, when the nitrogen quantity dwindles, or when poor nitrogen sources exist, Gln3 and Gat1 gradually localize in the nucleus, where they bind to target promoter and activate

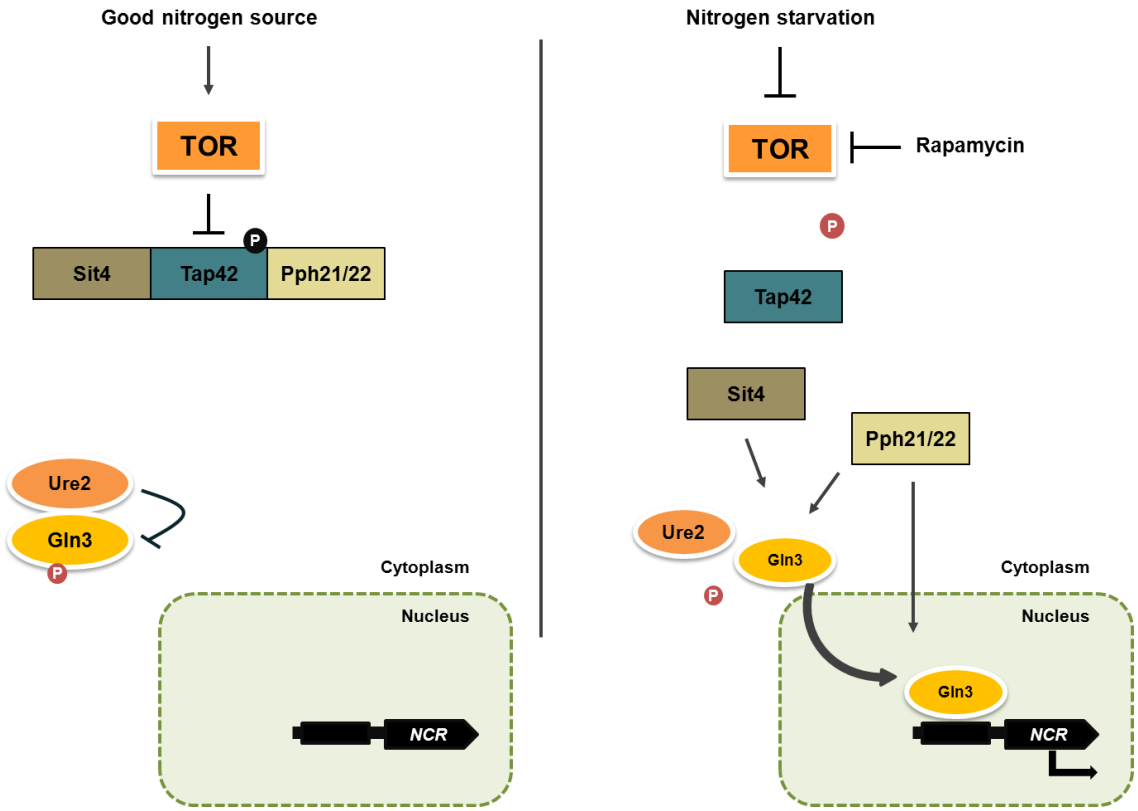


Figure 2.4 Regulation of nitrogen catabolic repression genes by GATA factors.

transcription of NCR-sensitive genes, required to transport and degrade poor nitrogen sources [15].

The nuclear localization of GATA factors is regulated by TORC1, Ure2, Sit4 and PP2A (Fig 2.4) [18,46,47]. Rapamycin treatment or nitrogen starvation condition down-regulate TORC1 activity [44]. These conditions activate and release Tap42-Sit4 or Tap42-PP2A phosphatases from TORC1. Activated Sit4 and PP2A phosphatases dephosphorylate Gln3, permitting it to dissociate from Ure2, enter the nucleus and activate transcription of NCR genes (Fig 2.4) [44].

2.2.3. Regulation of GATA factor-mediated transcription by PP2A-related phosphatases

Dephosphorylation and localization of GATA factors are regulated by downstream serine/threonine phosphatases Sit4 and PP2A [18]. Sit4 is a type 2A-like phosphatase catalytic subunit which is active in a complex with the phosphor-protein, Tap42 (Table 2.1). PP2A phosphatase is composed of a catalytic subunit, Pph21 or Pph22 complexes with a scaffold subunit, Tpd3, and one of two regulatory specificity subunits, Cdc55 or Rts1. Pph21 or Pph22 can also form complexes with Tap42 (Table 2.1) [48]. Sit4 and each PP2A associate with the phosphorylated form of Tor-associated protein, Tap42 [18]. TORC1 phosphorylates Tap42 under conditions of nitrogen excess. In a good nitrogen condition, the Tap42-phosphatase complexes are bound to TORC1 and are inactive. During nitrogen starvation or inhibition of TORC1 by rapamycin, the Tap42-phosphatase complexes are released from TORC1 and thereby become transiently active until they in turn dissociate into their inactive components, Tap42 and Sit4, or

Table 2.1 PP2A and PP2A-like phosphatase in *S. cerevisiae.*

Phosphatases	Function
Pph3	Catalytic subunit of protein phosphatase PP4 complex
Tip41	Tip41 regulates protein phosphatase 2A
Ptc1	Type 2C protein phosphatase
Tap42-Pph21/22	Essential protein complex involved in the TOR signaling pathway
Sit4	Type 2A-related serine/threonine phosphatase
Pph21/22-Tpd3-Cdc55	Cdc55 : It has multiple roles in mitosis and protein biosynthesis
Pph21/22-Tpd3-Rts1	Rts1 : B-type regulatory subunit of protein phosphatase 2A (PP2A)

Pph21/22 phosphatase catalytic subunits. These activated phosphatases contribute the nuclear localization and NCR promoter binding of GATA factors (Fig 2.4) [18].

2.3. Promoter engineering

2.3.1. General concept of promoter engineering

Transcription of gene is controlled by various factors in the organism, including transcription factors, various RNA polymerase-associated factors, promoter strength and environmental conditions [49]. Obviously, gene expression of mRNA levels may not always correspond with enzyme activity given at protein level of regulation, which may also be present in the cell. Nevertheless, synthetic control of gene expression is important in metabolic engineering fields. Specifically, optimized expressions of key pathway enzymes can support and maximize product biosynthesis. The optimization of transcriptional regulation occurs at promoter elements that drive gene expression [49]. However, native promoters are limited in that they do not maximize the transcription levels that are achievable within an organism. To solve this problem, various efforts in promoter engineering have shown a great promise both in expanding the transcriptional capacity and in enabling tunable expression levels of specific genes [50,51].

Most metabolic engineering studies in *S. cerevisiae* have focused on the selection of appropriate enzymes because enzyme homologs from various organisms could act differently in terms of expression and activity in *S. cerevisiae* [52]. Therefore selection of proper promoters is also important for the optimization of yield and productivity [24]. Promoter is important in several aspects of cellular process. Firstly, the transcription level of specific protein gene is determined by the promoter strength [49]. Secondly, promoters could be regulated and work differently under different growth

conditions [49]. Therefore many researchers have attempted to control gene expression through promoter engineering.

2.3.2. Example of promoter selection in metabolic engineering applications

Promoter selection is often a key component of the metabolic engineering. *E. coli* strains are selected as a host system for the overexpression of heterologous proteins [53]. In such applications, high strength and tightly controlled promoters are generally required to maximize protein production and reduce toxicity during growth phase. The end result is that only a few promoters are used for protein production despite hundreds of *E. coli* promoter sequences have been elucidated, including very high strength phage-derived promoter systems based on the T7 RNA polymerase and the P_L temperature-regulated phage promoter systems [53]. Sometimes, the abnormally high transcriptional capacity of these systems creates an excessive metabolic load on the *E. coli* host that decreases product formation in other metabolic engineering applications [53].

Many native promoters have been well characterized in *S. cerevisiae*. For example, strong endogenous constitutive promoters (including P_{ADH}, P_{TEF}, P_{HXT}, and P_{GPD}) or galactose-inducible promoter; P_{GAL} are usually employed for metabolic engineering application including the production of isoprenoids, butanol and various other metabolites [54,55,56,57,58]. However these promoters have several disadvantages for industrial application including limited inducible promoters, lack of promoter optimization and excessive high expression levels.

2.3.3. Promoter engineering strategy

Promoter engineering has been an essential tool in basic and applied biological research for the development of strains engineered to produce toxic proteins or metabolites [50]. However, toxic, expensive or inconvenient inducers using inducible promoters can be a limitation on their application. Best inducible promoter for the industrial application of yeast must be tightly regulated, express at proper levels after induction, inexpensive to induce, and easy to control. However, it is very difficult to satisfy all of these requirements. Doxycycline, galactose, copper ions and heat induced promoters in *S. cerevisiae* are actually leaky, inconvenient to use, and require expensive, toxic, or difficult-to-provide inducers [24].

From these reason, various strategies have been attempted to control gene expression via promoter engineering including error-prone PCR, saturation mutagenesis of nucleotide spacer regions, hybrid promoter engineering and direct modification of transcription factor binding sites.

Error-prone PCR inserts random mutations into specific DNA regions. Error-prone PCR performs to an entire promoter region; mutations occur all over the consensus and spacer regions of promoter, and lead to a different function [59,60]. This strategy is proper to novel promoter variants. This proper selection technique allows the isolation of promoters driving a wide range in gene expression. Also, the advantage of this approach is relatively easy to generate or improve specific promoters. Saturation mutagenesis of nucleotide spacer regions focuses on retaining consensus regions of the promoter regions by mutating variable regions. This strategy has been successfully applied to yeast promoter for synthetic library construction [52] .

Recently, hybrid promoter engineering have been conducted by using several well-known upstream activation sequence and endogenous core promoter in *S. cerevisiae* [33]. Synthetic hybrid promoters contain a core promoter region fused to a single upstream activating sequence or multiple tandem UAS repeats that modulate promoter strength. These upstream activation sequence regions recruit transcription factors to increase the transcriptional activity of the core promoter. Thus, the UAS and core promoter regions perform as modular synthetic parts that can be combined to produce various types of UAS-core promoters. In this concept, the strength of hybrid promoter can be elevated by either utilizing a stronger core promoter or by increasing the number of UAS repeats for the binding of transcription factor. Previously, synthetic hybrid galactose-inducible promoters showed a nearly 50-fold range of expression levels and additionally increased the transcriptional capacity of the original Gal1 promoter by about 20% [33]. This approach demonstrates that endogenous promoters in yeast can be enhanced simply through the addition of upstream activation elements to original core promoters.

To summarize, error-prone PCR mutagenesis method enables promoter engineering by randomly mutating DNA around or within transcription factor binding site on target promoters. And mutagenesis of nucleotide spacer regions enables promoter control by mutating DNA around conserved DNA motifs. Hybrid promoter engineering relies on this assumption by fusing tandem UAS elements to fine-tune the promoter strength.

Chapter 3.

Materials and methods

3.1. Yeast strains, media, and growth conditions

Yeast strains used in this work are listed in Table 3.1. Yeast strains were manipulated by PCR-mediated gene targeting method as previously described [61]. Construction of genomic TAP-tagged strains was performed as previously described [62]. Yeast cells were grown in YPD (1% yeast extract, 2% bacto-peptone, and 2% dextrose) or synthetic minimal medium (0.17% yeast nitrogen base without amino acids, 2% glucose, and 0.5% ammonium sulfate) supplemented with auxotrophic amino acids (histidine, leucine and methionine) and uracil when necessary or yeast nitrogen base (YNB) media containing 0.1% glutamine, ammonium sulfate, proline or urea as a sole nitrogen source. Rapamycin (Tecoland, USA) was dissolved in DMSO at a concentration of 1 mg/ml and used at a final concentration of 0.2 μg/ml.

3.2. Plasmids

Plasmid pRS416TEF-*ARO80-TAP* was generated by cloning a 3.4 kb PCR fragment containing *ARO80-TAP* ORF from BY4741 *ARO80-TAP* strain into SpeI and XhoI sites of pRS416TEF. pRS426ADH-*EGFP* vector was generated by cloning a 0.75 kb PCR fragment containing *EGFP* ORF into SpeI and BamHI sites of pRS426ADH. PCR-amplified *ARO80* ORF was cloned into BamHI and SalI sites of pRS426ADH-*EGFP*, resulting in pRS426ADH-*EGFP-ARO80*. *ARO80* gene was amplified by PCR with reverse primer containing a HA epitope sequence, and then cloned into BamH and SalI sites of pRS415ADH vector, resulting in pRS415ADH-*ARO80-HA* vector.

Tabel 3.1 Strains used in this study.

Strain	Pertinent genotype	Genotype	Source
JHY150	BY4741	BY4741 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0	EUROSCARF
JHY151	*aro80Δ*	BY4741 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 *aro80Δ*::KanMX6	EUROSCARF
JHY152	*gln3Δ*	BY4741 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 *gln3Δ*::KanMX6	EUROSCARF
JHY153	*gat1Δ*	BY4741 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 *gat1Δ*::KanMX6	EUROSCARF
JHY154	*gln3Δgat1Δ*	BY4741 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 *gat1Δ*::KanMX6 *gln3Δ*::HIS3MX6	This study
JHY155	*gzf3Δ*	BY4741 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 *gzf3Δ*::KanMX6	EUROSCARF
JHY156	*dal80Δ*	BY4741 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 *dal80Δ*::KanMX6	EUROSCARF
JHY157	*sit4Δ*	BY4741 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 *sit4Δ*::KanMX6	EUROSCARF
JHY158	*ure2Δ*	BY4741 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 *ure2Δ*::KanMX6	EUROSCARF
JHY159	*pph21Δ*	BY4741 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 *pph21Δ*::KanMX6	EUROSCARF
JHY160	*pph22Δ*	BY4741 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 *pph22Δ*::KanMX6	EUROSCARF
JHY161	*pph21Δpph22Δ*	BY4741 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 *pph21Δ*::KanMX6 *pph22Δ*::URA3	This study
JHY162	*tpd3Δ*	BY4741 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 *tpd3*Δ::URA3	This study
JHY163	*cdc55Δ*	BY4741 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 *cdc55*Δ::URA3	This study
JHY164	*rts1Δ*	BY4741 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 *rts1Δ*::KanMX6	EUROSCARF
JHY165	*rts1Δcdc55Δ*	BY4741 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 *rts1Δ*::KanMX6 *cdc55Δ*::URA3	This study
JHY166	*pde2Δ*	BY4741 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 *pde2Δ*::KanMX6	EUROSCARF
JHY167	*ira2Δ*	BY4741 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 *ira2Δ*::KanMX6	EUROSCARF
JHY168	*wsc1Δ*	BY4741 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 *wsc1Δ*::KanMX6	EUROSCARF
JHY169	*rom2Δ*	BY4741 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 *rom2Δ*::KanMX6	EUROSCARF
JHY170	*sho1Δ*	BY4741 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 *sho1Δ*::KanMX6	EUROSCARF
JHY171	*ssk1Δ*	BY4741 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 *ssk1Δ*::KanMX6	EUROSCARF
JHY172	*GAT1*-TAP	BY4741 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 *GAT1-TAP His3MX6*	Gaemmaghami et al., 2003
JHY173	*GAT1*-TAP *aro80Δ*	BY4741 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 *aro80Δ::KanMX6 GAT1-TAP His3MX6*	This study
JHY174	*GLN3*-TAP	BY4741 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 *Gln3-TAP His3MX6*	Gaemmaghami et al., 2003
JHY175	*GLN3*-TAP *aro80Δ*	BY4741 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 *aro80Δ::KanMX6 Gln3-TAP His3MX6*	This study
JHY176	*gln3Δgat1Δ*	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 *gat1Δ*::KanMX6 *gln3Δ*::HIS3MX6	This study
JHY177	*gap1Δagp1Δ*	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 *gap1Δ*::KanMX6 *agp1Δ*::HIS3MX6	This study
JHY178	*tat2Δ*	BY4741 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 *tat2Δ*::KanMX6	EUROSCARF
JHY179	*bap2Δ*	BY4741 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 *bap2Δ*::KanMX6	EUROSCARF
JHY180	*bap3Δ*	BY4741 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 *bap3Δ*::KanMX6	EUROSCARF
JHY181	*gap1Δ*	BY4741 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 *gap1Δ*::KanMX6	EUROSCARF
JHY182	*agp1Δ*	BY4741 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 *agp1Δ*::KanMX6	EUROSCARF

3.3. Microscopic analysis of protein localization

To localize EGFP-Aro80, *aro80Δ* cells containing pRS426ADH-*EGFP-ARO80* plasmid were cultured in minimal medium lacking amino acids in the absence or presence of tryptophan (50 μg/ml) until early log phase. Fluorescence signals were detected by fluorescence microscopy (Nikon ECLIPSE Ti-U), and localization of nuclei was detected by staining DNA with 2.5 μg/ml of 4, 6 diamidino-2-phenylindol (DAPI).

3.4. RNA preparation and quantitative RT-PCR (qRT-PCR)

Total RNA was isolated from yeast cells and the relative amount of specific mRNA was determined by qRT-PCR. Briefly, 2 μg of total RNA was subjected to reverse transcription in a 30 μl reaction mixture containing 200 U of MLV reverse transcriptase (M-biotech, Inc., Korea) and 0.1 mg of oligo-dT (M-biotech, Inc., Korea) at 42℃ for 60 min. Reaction mixture containing 1 μl cDNA, 1X SYBR master mix (Roche Diagnostics, Germany), and gene-specific primers was subjected to qPCR reaction with 45 cycles of 95℃ for 10 s, 55℃ for 20 s and 72℃ for 20 s using Roche LightCycler 480 real-time PCR system (Roche Diagnostics, Germany). Primer sequences used for qRT-PCR are shown in Table 3. 2.

3.5. Chromatin immunoprecipitation (ChIP)

ChIP experiment was conducted as previously described with some modifications [20]. Cells were cross-linked with 1% formaldehyde for 20 min and quenched with glycine at a final concentration of 125 mM for 5 min at 25℃. Cells were washed twice with cold TBS [20 mM Tris-HCl (pH7.5) and 150 mM NaCl]. Cells were resuspended in 800 μl lysis buffer [50 mM HEPES-KOH (pH7.5), 140 mM NaCl, 1 mM EDTA, 1% Triton-X 100, 0.1% Na-deoxycholate, 1 mM PMSF, and 1 μl protein inhibitor cocktail solution]. Glass beads were added to each sample, and then cells were broken by vortexing. Lysates were sonicated seven times for 20 sec with amplitude set at 23% and centrifuged twice at 13,200 rpm for 10 min. And then 20 μl of 50% slurry Ig-G agarose bead was incubated with 650 μl lysate at 4℃ for 3 hr. Beads were washed twice in lysis buffer, twice with high salt lysis buffer [50 mM HEPES-KOH (pH7.5), 500 mM NaCl, 1 mM EDTA, 1% Triton-X 100, 0.1% Na-deoxycholate, 1 mM PMSF, and 1 μl protein inhibitor cocktail solution], twice with wash buffer [1 mM Tris-HCl (pH 8.0), 250 mM LiCl, 0.5% NP40, 0.5% % Na-deoxycholate, and 1 mM EDTA], twice with TE buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA]. DNA was eluted by adding 100 μl of elution buffer [50 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 1% SDS] and incubating at 65℃ for 10 min. Elute was transferred to a fresh tube and pooled with 150 μl of elution buffer. 100 μl elution buffer was added to 50 μl of lysate for input. Input (IN) and immunoprecipitated (IP) samples were incubated at 65℃ for overnight, and then DNA was purified using Qiagen PCR purification kit in 55 μl of distillated water. Concentrations of the target promoter DNA fragment in IP and IN

samples were analyzed by qRT-PCR, and *[IP]/[IN]* values were calculated. Primer sequences used for ChIP are shown in Table 3.3.

3.6. Protein purification

In order to purify GST-Aro80 fusion protein from *E. coli* (Rosetta gami2 (DE3) pLysS), competent cells were transformed with pGEX-4T-1-Aro80 plasmid, and the protein were induces with 1 mM IPTG for 3 hr. GST-Aro80 fusion protein was purified using glutathione-agarose (Novagen). The purified GST-Aro80 was stored at -70℃ after dialysis against dialysis buffer containing 50 mM Tris-HCl (pH8.0) and 10% glycerol.

3.7. Western blotting

Cells were washed twice with TBS buffer [20 mM Tris-HCl (pH7.5) and 150 mM NaCl]. Cells were resuspended in 300 μl lysis buffer [50 mM Tris-HCl (pH7.5) and 150 mM NaCl, 0.1% NP40, 1 mM PMSF, and 1 μl protein inhibitor cocktail solution]. Glass beads were added to each sample, and then cells were broken by vortexing. Lysates were centrifuged twice at 13,200 rpm for 10 min. The samples were boiled after addition of SDS sample buffer [50mM Tris (pH7.5), 1mM EDTA, and 1% SDS], and then subjected to 7% SDS-PAGE. Aro80-TAP and Aro80-HA proteins were detected by immunoblotting with mouse anti-IgG-HRP antibody and high affinity rat anti-HA antibody, respectively.

3.8. Electrophoretic mobility shift assay (EMSA)

PCR products (10 ng) of *ARO9* promoters were radio-labeled with T4 polynucleotide kinase in the presence of [γ-32P] dATP and separated from unreacted DNA using Probe-Quant™ G-50 Micro columns (GE Healthcare, USA). The labeled probes were incubated with GST-tag purified Aro80 at room temperature. The total volume is 20 μL containing 20 mM HEPES (pH 7.5), 25 mM potassium acetate, 5% w/v glycerol, 100 mM KCl, 0.05 mM EDTA, 2 mM $MgCl_2$, 0.5 mM DTT, 0.05% Nonidet P-40 and 0.4 μg poly(dI-dC). Protein-bound DNA and free DNA were resolved on 5% acrylamide gels in 0.5X TBE buffer (45mM Tris-borate and 1mM EDTA) at RT. The gels were dried and exposed overnight. The analysis was done with Typhoon 8600 scanner (GE Healthcare).

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

To detect intracellular metabolites, samples were prepared as previously described with some modifications [63,64,65], and analyzed by UPLC/QQQ-MS. Liquid chromatography was performed on a Accela 1250 UPLC™ system (ThermoFisher scientific, USA), using 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℃, 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 cycle time was 35 min, with a flow rate of 400 μ /min and an injection volume of 10 μL using partial-loop mode. Mass spectrometry was performed using a TSQ quantum Access Max triple quadrupole mass spectrometer (ThermoFisher scientific, USA). Ionization was performed in the positive and negative switching 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℃; source temperature, 270℃; and desolvation temperature, 350℃. 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 for triple quadrupole mass spectrometry

3.10. Gas chromatography

To determine metabolite concentration, 1 ml of culture supernatants were collected and filtered through a 0.22 μm syringe filter. GC analyses were performed in a Varian GC-450 gas chromatograph equipped with a DB-WAX capillary column (30 m×0.32 mm ID, 0.25 μm film thickness, Agilent) and a flame ionization detector (300 °C). The following temperature program was used: isotherm at 40 ℃ for 5 min, 10 ℃/min ramp to 170 ℃, 30 ℃/min ramp to 230 ℃ and isotherm at 230 ℃ for 5 min. Acetoin and (2R,3R)-2,3-butanediol for standard solution were purchased from Sigma-Aldrich.

3.11. Measurement of GFP fluorescence intensity

The GFP fluorescence intensity of the cells harvested from shake flasks was measured using a microplate reader. Briefly, cells were harvested and resuspended with phosphate-buffered saline (PBS) to an optimal density between 0.1 and 0.2 at 600 nm, and transferred to 96 well plate. Microplate reader analysis was performed using Tecan micro-plate reader, and the mean fluorescence intensity distribution of each clonal population was calculated.

Tabel 3.2 qRT-PCR primer sequences used in this study.

Primer	Sequence
ARO9F	GGAATTTCGATAGACCTGACGA
ARO9R	GGAAGCTTCAATCAACTGATC
ARO10F	ACTCAATATACGAACGAAACA
ARO10R	CTCCTCGAATTCTTAAGTTTG
ARO80F	GAGATGGCACCACTCCTCTAGAG
ARO80R	CAAGCCCGATATCTTCACTAGC
HSP26F	GGTCAAGGTCAAGGAGAGCAG
HSP26R	GTGGTTCTTACCATCCTTCTG
DAL5F	CTGGCTGGTGCTTACCTGTGG
DAL5R	GGGAACCCTCTCTCGGCAGC
CTT1F	CAAAGGGATAGTTCTTGACG
CTT1R	GACCAAGTCTTGGCATAACC
ENO1F	CTATCGAAAAGAAGGCTGCC
ENO1R	CGTGGTGGAAGTTTTCACCAGC
ACT1F	CTGCCGGTATTGACCAAAC
ACT1R	GGTGAACGATAGATGGACC
CAR1F	GTGCTCCTATGACGTCGATG
CAR1R	GCAATGGCGCAACCTGCAG
PUT1F	CGATCGTATCATCACTCAAG
PUT1R	GCGCCATGGTTGGTAATTAG
TBP1F	TATAACCCCAAGCGTTTTGC
TBP1R	GCCAGCTTTGAGTCATCCTC

Tabel 3.3 ChIP primer sequences used in this study.

Primer	Sequence
pARO9F	GTGACTCCGCGCAGGGCTGT
pARO9R	GTTTCCTACCCCAATGATGG
pARO10F	GTACACCTCATGTAGCTTCC
pARO10F	GTGAGCTGCCGCTGTAGTGACG
pARO80F	GAGTCCTTCTTAGTAATACATGAAGG
pARO80F	GAGGATAAAGCAGTGCTTAATG
pCTT1F	GCCAAGACCGCGGACGGC
pCTT1R	CCCTTCTGGATCGGCGACG
pGAP1F	GACCTCATGCAGCAAAGTCA
pGAP1R	CCGGTTGCTCCAGAAGATAA
pDAL5F	CGAGGAGCTATCATTTGCTG
pDAL5R	ATCTTTTGCCCCGATAATCC

Chapter 4.

Activation mechanism of Aro80 by ligands

4.1. Introduction

Yeast cells can use aromatic amino acids as a sole nitrogen source [2]. Aromatic amino acids are catabolized to the corresponding alcohols through Ehrlich pathway, which consists 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 [5]. Aro9 and Aro10 act as transaminase and decarboxylase, respectively, in the Ehrlich pathway. Although Aro9 and Aro10 have been shown to have broad substrate specificities, expression of *ARO9* and *ARO10* genes is highly induced by aromatic amino acids, suggesting their important roles in aromatic amino acid catabolism. Aro80, a member of the Zn2Cys6 proteins, activates expression of the *ARO9* and *ARO10* genes in response to aromatic amino acids (Fig 4.1) [2]. The Aro80 binding site, consisting of four CCG repeats spaced by 7 bp, was initially identified in the promoters of *ARO9* and *ARO10* [66]. Computational analysis of Aro80 ChIP-chip data has revealed a putative binding sites of Aro80, WWNCCGANRNWNNCCGNRRNNW, which is also identified in the promoters of *ARO80* itself and *ESBP6* encoding a protein homologous to a monocarboxylate permease (Fig 4.2) [10]. In addition to the induction by aromatic amino acids, expression of *ARO9* and *ARO10* is regulated by nitrogen source. It has been shown that *ARO9* is induced by poor nitrogen source (urea) and rapamycin treatment, suggesting that its expression might be under the control of NCR. Previously, it has been suggested that GATA factors might regulate *ARO9* indirectly by permease-dependent regulation of cellular inducer levels [2].

In this chapter, the regulation mechanism of Aro80 target genes via various ligands was elucidated. Here it was confirmed that tryptophol and methionine as well as

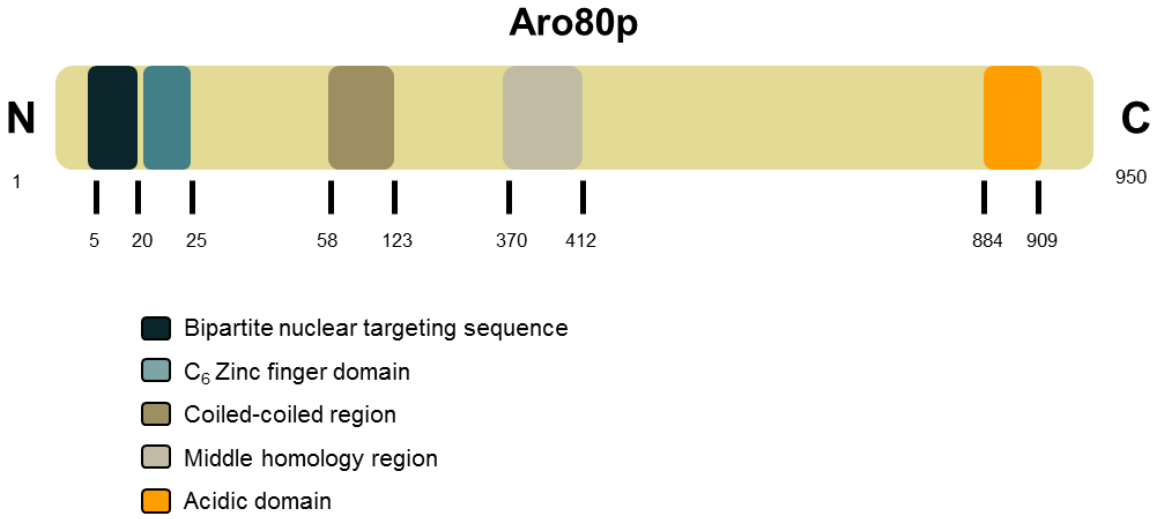


Figure 4.1 The structure of transcription factor Aro80.

ARO9 -171 GCATTG**CCG**ATGCTTA**CCG**AGATTTG**CCG**CGGATAA**CCG**AAC -130

ARO10 -349 GGATAA**CCG**CGGATAG**CCG**TCATTTA**CCG**AAAATTG**CCG**AGG -308

ARO80 -148 TTCTAT**CCG**ATGATAA**CCG**AGATAAATGAAGATAGTAACTAA -107

ESBP6 -371 CGCTTG**CCG**ACGATAA**CCG**AGATAACGTATCTTTGCCGCGGG -330

The consensus binding site of Aro80: WWN**CCG**ANRNWNN**CCG**NRRNNW

Figure 4.2 The promoter sequences of Aro80 target genes.

CCG triplets, the binding site of Aro80, are underlined in bold

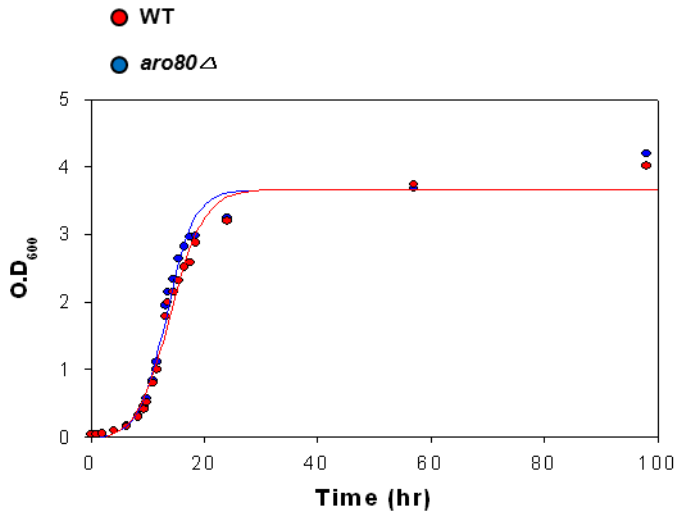
aromatic amino acids activate the expression of *ARO9* and *ARO10* in an Aro80 dependent manner. Transcription factor Aro80 always exists in the nucleus irrespective of the inducer availability. Also, the localization of Aro80 does not regulate the activity of Aro80. Furthermore, it was found that the binding affinity of Aro80 to target promoter does not change the existence of ligands *in vivo* and *in vitro*. From these results, it suggests that Aro80 is constitutively bound to its target promoters and becomes activated in the presence of aromatic amino acids.

4.2. Aro80-dependent expression of *ARO9* during growth

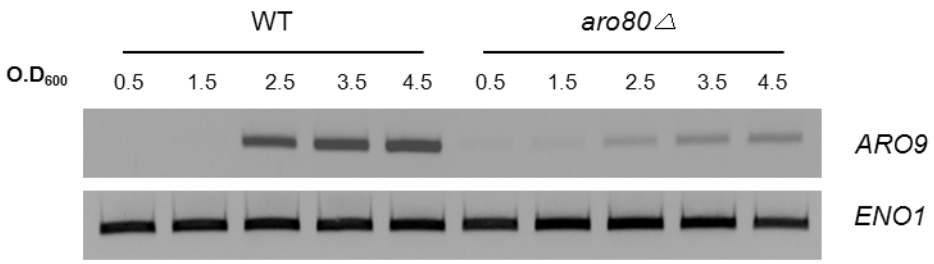
It was previously reported that the expression of *ARO9* and *ARO10* genes is regulated by transcription activator Aro80 [2]. Yeast cells cannot use a tryptophan as a sole nitrogen source in the *ARO80* deletion strain because of restriction of expression of *ARO9* and *ARO10*. First of all, it was investigated whether the transcription level of *ARO9* genes is regulated in growth phase. Yeast wild type and *aro80Δ* cells were cultured in synthetic complete media and were harvested at five different time points during growth phase.

There was no difference in growth between wild type and *aro80Δ* strains (Fig 4.3 A). And then transcription levels of *ARO9* were compared between wild type and *aro80Δ* cells through RT-PCR. From results, it was found that the expression of *ARO9* gradually increased by growth phase and the expression level of *ARO9* highly depended on transcription factor Aro80 (Fig 4.3 B).Next, the expression of *ARO9* and *ARO80* genes at different time points in SLAD media was observed (Fig 4.3 C). SLAD media contains very low ammonium concentration as a sole nitrogen source. Therefore, ammonia in SLAD media as a nitrogen source was rapidly decreased by cell growth. Interestingly, the expression of *ARO9* was gradually increased and then the highly expressed transcript of *ARO9* was maintained up to 24 h (Fig 4.3 C). On the other hand, expression of *ARO80* was rapidly decreased after 8 h (Fig 4.3 C). These results suggest two possibilities. First, expressed mRNA of *ARO9* is very stable. Second, other regulators might be involved in the expression of *ARO9* under nitrogen depletion condition like SLAD media.

A


WT
aro80⊿
O.D600
Time (hr)
0
1
2
3
4
5
0
20
40
60
80
100

B


WT
aro80⊿
O.D600
0.5
1.5
2.5
3.5
4.5
0.5
1.5
2.5
3.5
4.5
ARO9
ENO1

C

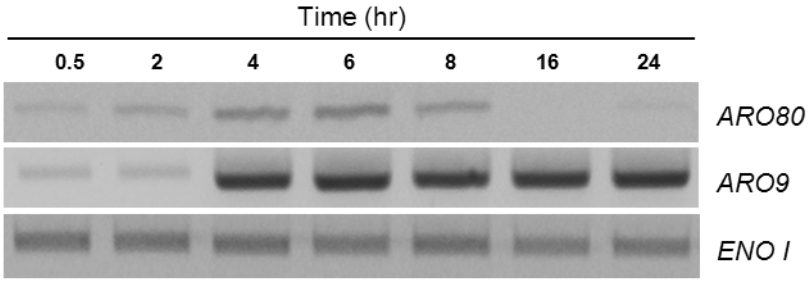

Time (hr)
0.5
2
4
6
8
16
24
ARO80
ARO9
ENO I

Figure 4.3 Aro80-dependent expression of *ARO9* during growth.

A. Growth curve of WT and *aro80Δ* cells grown in SD media.

B. Aro80-dependent expression of *ARO9*. mRNA expression levels were detected by RT-PCR. *ENO1* was used as a loading control.

C. Expression levels of *ARO9* and *ARO80* genes by time points. mRNA expression levels were detected by RT-PCR. *ENO1* was used as a loading control.

4.3. Aro80-dependent expression of *ARO9* and *ARO10* by aromatic amino acids

Previously, it was known that aromatic amino acids activated the transcription of *ARO9* [2]. However, Trp concentration dependent-expression of *ARO9* was not elucidated. Therefore, it was investigated whether expression levels of *ARO9* and *ARO10* were regulated by tryptophan concentrations. Yeast cells were cultured in various concentrations of tryptophan medium as a sole nitrogen source. And then the growth rates and the expression levels of *ARO9* and *ARO10* were observed. Growth rates of early exponential phase are noticeably decreased by the increase of tryptophan concentrations (Fig 4.4 A). On the other hand, the final cell density increased with increasing tryptophan concentrations, which suggest that more tryptophan was used as a nitrogen source (Fig 4.4 A). Furthermore, expression levels of *ARO9* and *ARO10* genes were increased by increase in the tryptophan concentrations (Fig 4.4 B). These results suggest that yeast cells can use tryptophan as a sole nitrogen source via the increase of transcription of tryptophan degradation enzymes, *ARO9* and *ARO10*.

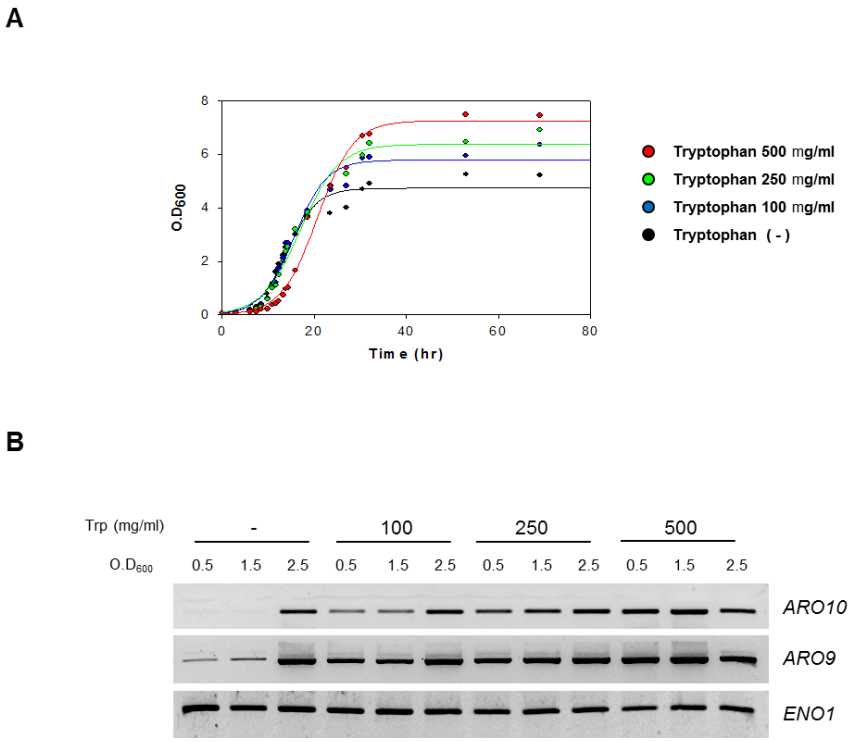


Figure 4.4 Differences in the growth rate and expression levels of *ARO9* and *ARO10* in *S. cerevisiae* with respect to tryptophan concentrations.

A. Growth curve of *S. cerevisiae* in minimal medium with various tryptophan concentrations as a sole nitrogen source.

B. Expression of *ARO9* and *ARO10* depending on Trp concentration. mRNA expression levels were detected by RT-PCR. *ENO1* was used as a loading control.

4.4. Tryptophan independent expression of *ARO80* and *ESBP6*

Aro80, a member of the Zn_2Cys_6 transcriptional regulator, activates expression of the *ARO9* and *ARO10* genes in response to aromatic amino acids and tryptophol. It was previously reported that the binding site of Aro80, consisting of four CCG repeats spaced by 7 base pair, was initially identified in the promoters of *ARO9* and *ARO10* (Fig 4.2). Also, the 36 bp sequence contains a UAS element necessary and sufficient for induced expression of the fusion gene in response to tryptophan [2]. Computational analysis of Aro80 ChIP-chip data has revealed a putative binding site of Aro80, WWNCCGANRNWNNCCGNRRNNW, which is also identified in the promoters of *ARO80* itself and *ESBP6* encoding a protein homologous to a monocarboxylate permease [10]. Therefore *ARO80* and *ESBP6* genes are potential target of Aro80. Especially, *ARO80* promoter contains conserved Aro80 binding sites, suggesting a potential auto-regulation of its own expression (Fig 4.2). Therefore, it was investigated whether expression of *ARO80* and *ESBP6* genes can be induced by aromatic amino acids. Whereas, expression of *ARO9* was induced by tryptophan as previously reported, and expression of *ARO80* and *ESBP6* was not affected by the availability of inducer, indicating that Aro80 might not activate expression of *ARO80* and *ESBP6* in response to aromatic amino acids (Fig 4.5). This result suggests a requirement of a more detailed study on the different properties between *ARO80* and *ARO9* promoters.

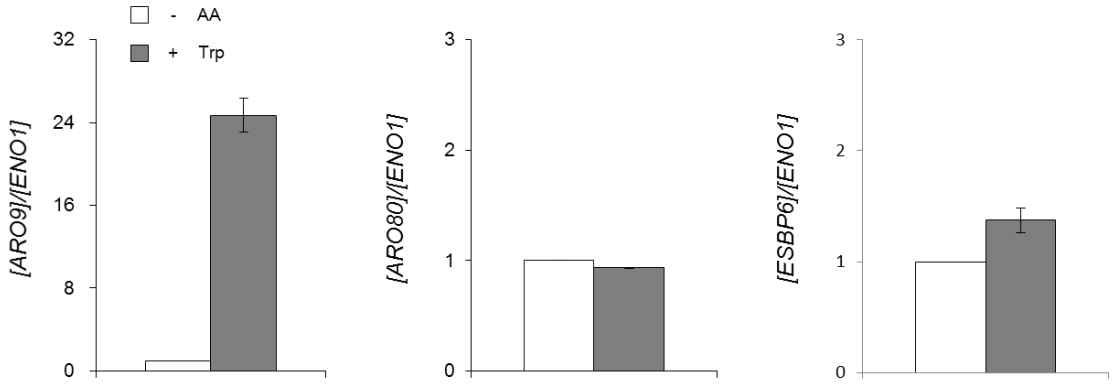


Figure 4.5 Expression levels of *ARO9, ARO80* and *ESBP6* by tryptophan.

BY4741 cells were grown in minimal medium lacking amino acids (− AA) or containing 50 μg /ml tryptophan (+ Trp) until early exponential phase. *ARO9, ARO80* and *ESBP6* mRNA levels were analysed by qRT-PCR normalized with *ENO1*. Each value represents the average ± standard deviation from three independent experiments.

4.5. Inducer-dependent regulation mechanism of Aro80

Environmental signaling can induce the rapid redistribution of transcriptional regulators from the cytosol to the nucleus by a number of different mechanisms. In order to perform their functions as transcription factors, zinc cluster proteins must be localized to the nucleus. Interestingly, it was well known that many zinc cluster proteins such as Put3, Oaf1, War1 and Leu3 constitutively localized within the nucleus with or without additional signals [67,68,69].

To understand the activation mechanism of Aro80, its cellular localization of EGFP-Aro80 depending on the inducer availability were examined. From result, EGFP-Aro80 was observed in the nucleus even in minimal medium lacking amino acids, and addition of tryptophan to the medium did not elicit any significant changes in Aro80 localization (Fig 4.6), suggesting that Aro80 always exists in the nucleus irrespective of availability of the inducer. Many zinc cluster proteins like Leu3, Put3, War1, Oaf1 and Lys14 are constitutively localized within the nucleus on a permanent basis. Moreover these regulators are also constitutively bound to their target promoters. For example, Put3 is a transcriptional regulator of proline utilization genes that encode enzymes required for proline catabolism [35,38,70,71]. Although transcriptional activator Put3 is always bound to target promoters, the activity of Put3 is regulated by direct interaction with proline [70,71,72]. Similarly, transcription factor Ppr1 regulates the transcription of pyrimidine biosynthetic pathway related genes. This transcription factor is constitutively bound to target promoters in an inactive state but is activated by a metabolic intermediate [36].

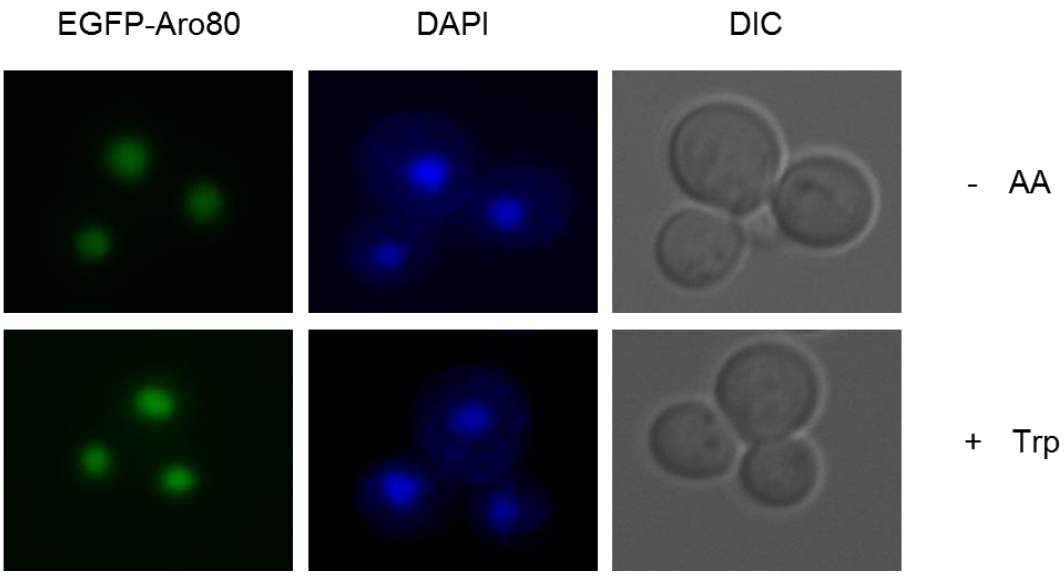


Figure 4.6 Cellular localization of EGFP-Aro80.

BY4741 cells expressing EGFP-Aro80 were grown in minimal medium lacking amino acids (−AA) or containing 50 μg/ml tryptophan (+Trp) until early exponential phase, and cellular localizations of EGFP-Aro80 and DAPI-stained nucleus were observed by fluorescence microscopy.

In addition, a transcription factor Leu3 is bound to target promoters of leucine biosynthesis genes. Leu3 is also activated in the presence of α-isopropylmalate, a leucine precursor [35].

Previous results of this study showed that Aro80, like other zinc cluster proteins, is also localized in nucleus. However interaction between Aro80 and target promoter have still not been elucidated. Therefore, the binding of Aro80 to *ARO9* promoter was observed. It was investigated whether the binding affinity of Aro80 to the target promoters was regulated by the existence of ligands. In order to observe interaction between Aro80 and *ARO9* promoter, electrophoretic mobility shift assays (EMSA) were performed using *E. coli* purified GST-Aro80 protein (Fig 4.7). From the result, it was found that the binding affinity of Aro80 to *ARO9* promoter increases in the concentration-dependent manner with GST-Aro80. And the binding affinity of Aro80 to *ARO9* promoter was not changed by tryptophol, phenylethanol, tyrosol, tryptophan, phenylalanine and tyrosine *in vitro* (Fig 4.7). It means that ligand is not important for target promoter binding of Aro80. Rather, ligands of Aro80 might regulate the activity of Aro80 for expression of *ARO9* and *ARO10*.

The binding of Aro80 to *ARO9* promoter was confirmed *in vivo* by chromatin immuno-precipitation (ChIP) experiments. Aro80-TAP could induce expression of *ARO9* and *ARO10* in response to tryptophan, indicating that the TAP-tagged Aro80 is functional (Fig 4.8 A, B). Similarly to the *in vitro* EMSA result, chromatin immunoprecipitation (ChIP) analysis showed no significant change in the amount of Aro80-TAP bound to the *ARO9* promoter by tryptophan (Fig 4.9). These *in vitro* and *in*

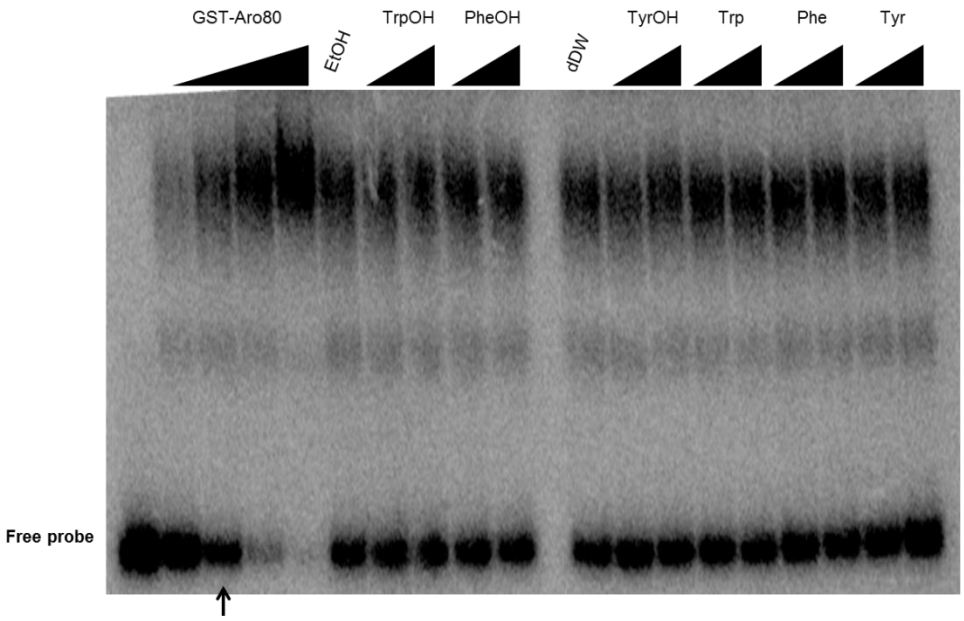


Figure 4.7 Binding affinity of Aro80 to the *ARO9* promoter in the absence or presence of ligands.

Binding of GST-Aro80 purified from *E. coli* to *ARO9* promoter was detected by electrophoretic gel mobility shift assay.

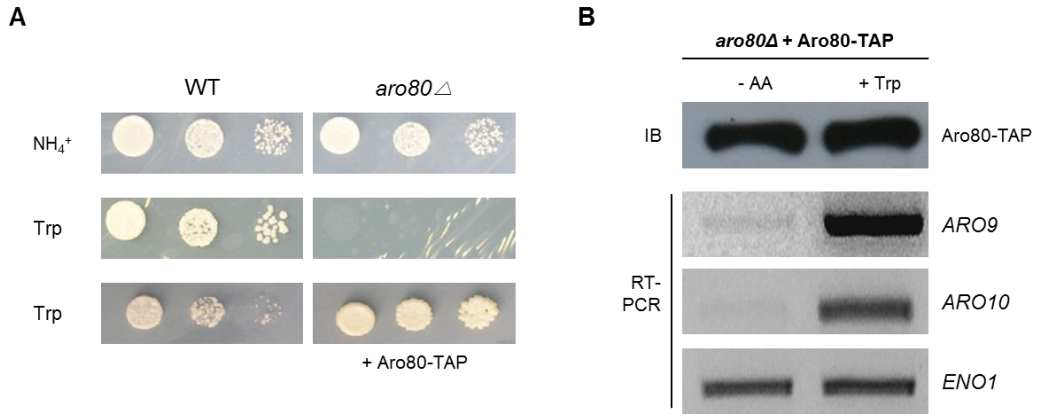


Figure 4.8 Functional test of over expressed Aro80-TAP strain.

A. Growth restoring of yeast cell by the complementation of Aro80-TAP. BY4741 *aro80Δ* cells harboring pRS416TEF-*ARO80-TAP* plasmid were grown in minimal medium containing ammonia or tryptophan plate as a sole nitrogen source.

B. Aro80-TAP is functional in tryptophan-dependent induction of *ARO9* and *ARO10*. BY4741 *aro80Δ* cells harboring pRS416TEF-*ARO80-TAP* plasmid were grown in minimal medium lacking amino acids and treated with 500 μg/ml tryptophan for 1 h. Aro80-TAP protein expression levels were detected by immunoblotting (IB) using anti-mouse IgG-HRP antibody, and the *ARO9* and *ARO10* mRNA levels were determined by RT-PCR. *ENO1* was used as a control.

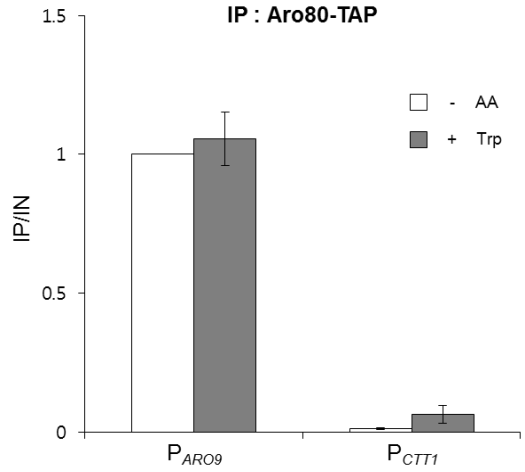


Figure 4.9 Binding affinity of Aro80 to *ARO9* promoter *in vivo.*

ChIP analysis of Aro80-TAP. BY4741 *aro80*Δ cells expressing Aro80-TAP were grown in minimal medium lacking amino acids and treated with 500 µg/ml tryptophan for 1 h. Aro80-TAP binding to the *ARO9* and control *CTT1* promoters was analysed by ChIP in the absence (−AA) or presence of tryptophan (+Trp). Concentrations of the target promoter DNA in the immunoprecipitated (IP) and input (IN) samples were quantified by qPCR. Results are expressed as relative IP/IN values ± standard deviations obtained from three independent experiments.

vivo results suggest that Aro80 is constitutively bound to its target promoters and becomes activated in the presence of aromatic amino acids.

4.6. Conclusions

In this chapter, it has been shown that the transcription of Aro80 target genes was activated by various ligands such as tryptophan, phenylalanine, tyrosine, methionine and tryptophol. Although *ARO9*, *ARO10*, *ARO80* and *ESBP6* of promoters have the consensus binding motif of Aro80, ligands could not activate the transcription of *ARO80* and *ESBP6*. Also, it has been confirmed that GFP-Aro80 was always localized in nucleus. In addition, this study found that Aro80 was constitutively bound to its target promoters *in vivo* and *in vitro* and became activated in the presence of aromatic amino acids.

Chapter 5.

Interplay of Aro80 and GATA activators in nitrogen source dependent expression of *ARO9* and *ARO10*

5.1. Introduction

Saccharomyces cerevisiae reprograms cellular metabolism in response to the quantity and quality of nitrogen sources available in the environment [1,41,73]. Yeast cells can use a wide range of compounds as nitrogen sources, but expression of genes for utilization of non-preferred nitrogen sources such as proline, allantoin, γ-aminobutyrate (GABA) is inhibited in the presence of preferred nitrogen sources such as glutamine and ammonia. This global nitrogen quality control is known as nitrogen catabolite repression (NCR) [1]. Transcription of NCR-sensitive genes encoding permeases and catabolic enzymes of the poor nitrogen sources is regulated by four GATA factors consisting of two activators, Gln3 and Gat1/Nil1, and two repressors, Gzf3/Nil2/Deh1 and Dal80/Uga43, which bind to GATAA sequences in the promoters [74,75]. When a good nitrogen source is available, Gln3 and Gat1 are sequestered in the cytosol. In the presence of a poor nitrogen source or upon depletion of a good nitrogen source, Gln3 and Gat1 translocate to the nucleus, leading to activation of the NCR-sensitive genes. Although the nitrogen-responsive regulatory mechanisms for Gln3 and Gat1 are not completely understood, TOR (target of rapamycin) kinase signaling pathway is in part implicated in the regulation of GATA factors [14,76]. TOR kinase, a conserved protein kinase from yeast to humans, controls a wide range of metabolic processes in response to nutrient availability. In *S. cerevisiae*, TOR exists in two distinct protein complexes, TORC1 and TORC2, of which TORC1 is inactivated by rapamycin. Rapamycin treatment mimics the effect of nitrogen limitation, resulting in nuclear localization of Gln3 and Gat1, and activation of NCR-sensitive [41]. TORC1-dependent regulation of Gln3 localization requires protein phosphatase 2A (PP2A)-like phosphatase [77]. Under nitrogen-rich conditions, active TORC1 phosphorylates Tap42, thus facilitating

a TORC1-Tap42-Sit4 complex formation, which inactivates Sit4 activity. When TORC1 is inhibited by rapamycin, Tap42-Sit4 is released from TORC1, and the activated Sit4 dephosphorylates Gln3, leading to its dissociation from Ure2 and nuclear localization [77]. Although rapamycin induces more prominent nuclear localization of Gat1 than Gln3, Sit4 is not absolutely required for rapamycin-dependent Gat1 localization [78]. In addition to Sit4, PP2A phosphatase functions as a downstream effector of TORC1. Active PP2A is composed of a catalytic subunit, Pph21 or Pph22, and a regulatory subunit, Cdc55 or Rts1, linked by a scaffolding subunit Tpd3. Pph21 or Pph22 also forms an active phosphatase complex with Tap42. It has been shown that PP2A is involved in rapamycin-dependent nuclear localization of Gln3 and Gat1 in glutamine or ammonia grown cells at the level of upstream and downstream of Ure2, respectively [79]. Furthermore, PP2A also plays a role in GATA factor binding to the nitrogen-responsive promoters upon rapamycin treatment by yet an unknown mechanism [80].

In this chapter, the novel regulatory mechanism for expression of *ARO9* and *ARO10* genes by nitrogen depletion condition was elucidated. Positive GATA factors Gln3 and Gat1 directly regulated the expression of *ARO9* and *ARO10*. And Aro80 was required for binding of Gat1 and Gln3 to the promoters of *ARO9* and *ARO10* upon rapamycin treatment, suggesting a dominant role for Aro80 in regulating its target genes under nitrogen depletion condition. Also it was observed that negative GATA factors were not involved in the expression of *ARO9* and *ARO10*.

5.2. The characteristic of promoter sequence of Aro80 target genes

GATA factors are responsible for nitrogen catabolite repression of genes encoding transports and enzymes required to scavenge poor nitrogen sources [1]. Expression of NCR-sensitive gene is regulated by the finely tuned and integrated system of the two transcription activators (Gln3 and Gat1) and two transcription repressors (Dal803 and Gzf3) [1]. Positive GATA factors bind to single GATAA sequence. On the contrary, negative GATA factors consist of leucine-zipper domains near their C-terminal and form homodimers and heterodimers [1]. Consistent with the formation of dimers, Dal80 binding to target promoters requires two GATAAG sequences. Gzf3 also binds to target promoters containing single GATAAG sequence in a GATAA sequence-dependent manner. Integration of the regulation of GATA activators and repressors is accomplished by their cross regulation [17].

All of Aro80 target gene promoters contain potential GATA binding sites. The promoter of *ARO9* and *ARO10* consist of a single GATAA sequence (Fig 4.2). In the case of promoters of *ARO80* and *ESBP6*, they have two GATAA and GATAAG sequence motif (Fig 4.2). This observation shows possibility that GATA factors are involved in the regulation of Aro80 target genes under poor nitrogen conditions.

5.3. Aro80, Gat1 and Gln3 are involved in rapamycin-dependent induction of Aro80 target genes

Previously, it has been suggested that GATA factors might regulate *ARO9* indirectly through regulation at cellular inducer levels via Gap1 and Agp1 amino acid permeases [2]. However, the presence of potential GATA factor binding sites next to the Aro80 binding sites suggests a direct role for GATA factors in the regulation of Aro80 target genes. Therefore, this issue was revisited and the roles of GATA activators were examined; Gln3 and Gat1, in regulation of Aro80 target genes upon TORC1 inhibition by rapamycin. In agreement with the previous report, rapamycin-dependent induction of *ARO9* and *ARO10* was almost abolished in *aro80Δ* strain in YPD medium (Fig 5.1 A). In addition, *gln3Δ* and *gat1Δ* strains also showed reduced induction of these genes upon rapamycin treatment (Fig 5.1 A). Rapamycin-dependent induction of these genes was further reduced in *gln3Δgat1Δ* double deletion mutant, confirming that both GATA factors are involved in activation of these genes. Expression of *ARO80* was also induced by rapamycin in a GATA factor-dependent manner although the induction fold was smaller than those of *ARO9* and *ARO10* (Fig 5.1 A). Since *ARO80* expression is independent of inducer levels, GATA factors might directly activate *ARO80* expression (Fig 5.1 A). The roles for GATA factors in the rapamycin-dependent induction of *ARO9* and *ARO10* might also include transcriptional activation of *ARO80* as well as the indirect activation of Aro80 as previously proposed.

Although Gat1 and Gln3 are well known to be regulated by TORC1 signaling pathway, the rapamycin-dependent induction of *ARO9* and *ARO10* genes was not completely abolished in *gln3Δgat1Δ*. Based on the fact that Aro80 plays an essential

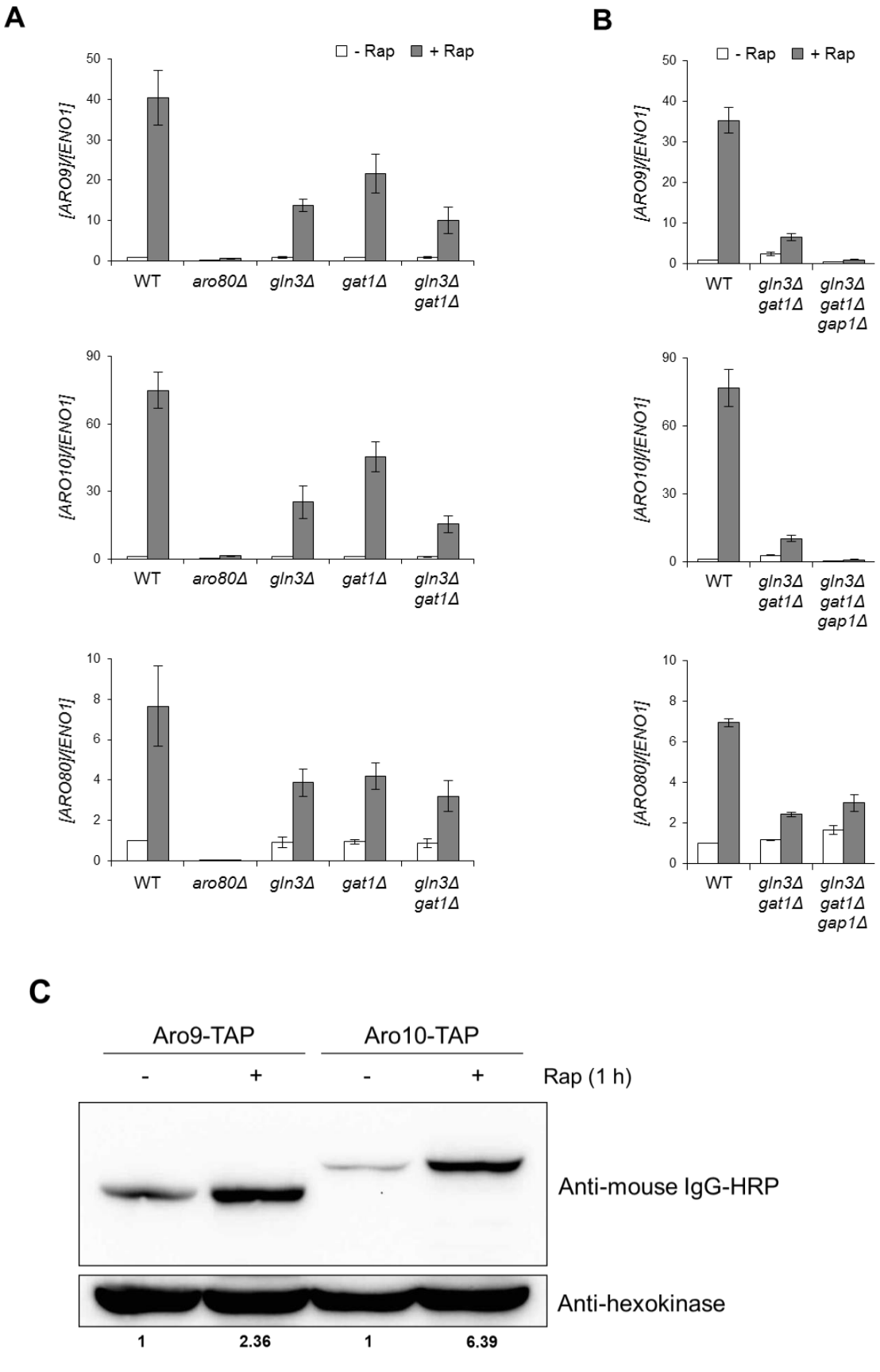

A
B
- Rap
+ Rap
[ARO9]/[ENO1]
[ARO10]/[ENO1]
[ARO80]/[ENO1]
WT
aro80Δ
gln3Δ
gat1Δ
gln3Δ gat1Δ
gln3Δ gat1Δ gap1Δ
C
Aro9-TAP
Aro10-TAP
Rap (1 h)
Anti-mouse IgG-HRP
Anti-hexokinase
1
2.36
1
6.39

Figure 5.1 Aro80, Gat1 and Gln3 are involved in rapamycin-dependent induction of Aro80 target genes.

A. Effects of Aro80, Gat1 and Gln3 on rapamycin-dependent induction of Aro80 target genes. Cells were cultured in YPD medium until early exponential phase and then treated with 0.2 μg /ml rapamycin (Rap) for 1 h. mRNA levels of *ARO9*, *ARO10* and *ARO80* were determined by qRT-PCR normalized with *ENO1*. Each value represents the average of three independent experiments.

B. Effect of Gap1 permease on the residual induction of Aro80 target genes in *gln3*Δ*gat1*Δ strain. WT, *gln3*Δ*gat1*Δ and *gln3*Δ*gat1*Δ*gap1*Δ strains were grown in YPD medium until early exponential phase and then treated with 0.2 μg./ml rapamycin (Rap) for 1 h. mRNA levels of *ARO9*, *ARO10* and *ARO80* were determined by qRT-PCR normalized with *ENO1*.

C. Protein expression levels of Aro9 and Aro10 upon rapamycin treatment. Aro9-TAP and Aro10-TAP stains were cultured in YPD medium until early exponential phase and then treated with 0.2 μg /ml rapamycin (Rap) for 1 h. Proteins levels of *ARO9* and *ARO10* were determined by western blot normalized with hexokinase.

role in rapamycin-dependent induction of *ARO9* and *ARO10*, the remaining induction in *gln3Δgat1Δ* might be mediated by Aro80. Stabilization of Gap1, a general amino acid permease, could be one of the mechanisms for the increase in amino acids uptake upon rapamycin treatment [44], leading to Aro80 activation. In agreement with this hypothesis, expression of *ARO9* and *ARO10* in *gln3Δgat1Δ* was diminished to basal levels in rapamycin-treated cells by additional deletion of *GAP1*. However, in line with the observation that *ARO80* is not induced by aromatic amino acids, *gln3Δgat1Δgap1Δ* did not show any significant difference in rapamycin-dependent *ARO80* expression compared with *gln3Δgat1Δ* (Fig 5.1 B). Therefore, the residual induction of *ARO80* in *gln3Δgat1Δ* is not mediated by the inducer-dependent activation of Aro80, suggesting the presence of other yet unidentified regulatory mechanisms. And in this study, Aro9 and Aro10 protein levels were confirmed by western blot (Fig 5.1 C). From result, expression levels of Aro9-TAP and Aro10-TAP were significantly increased by rapamycin treatment. Therefore rapamycin treatment contributes the increase of mRNA and protein levels of *ARO9* and *ARO10* in *S. cerevisiae*.

5.4. Screening of Aro80 upstream signal transduction pathway

Rapamycin-dependent induction of *ARO9* and *ARO10* was totally abolished in *ARO80* deletion strain. Therefore, it was hypothesized that the activation of Aro80 upon rapamycin treatment is mediated by TOR directly or via mediators [77]. In *S .cerevisiae*, TOR can mediate phosphorylation of yeast AGC kinases. Other non-AGC kinase proteins are also phosphorylated in a TOR-dependent manner, such as the type 2A phosphatase-associated protein 42 (Tap42) and Tip41, Gln3, Npr1 kinase and phosphatidylinositol-binding proteins Slm1/2 [77]. Table 5.1 shows TOR regulated AGC kinase or non-AGC kinase proteins including various phosphatases. And Table 5.2 shows various stress response pathway proteins.

Therefore, it was investigated whether TOR and stress response related proteins among these proteins were involved in the activation of Aro80 under rapamycin. From result, it was found that deletion of some kinases and phosphatase involved in TORC1 signaling pathway (*SCH9*, *SKY1*, *KSP1*, *RIM15*, *PPH3*, *PKH1* and *PKH3*) did not affect the expression of Aro80 target genes upon rapamycin treatment. Although this study cannot rule out the possibility that Aro80 is also a target of TORC1 signaling pathway, this research could not detect any nitrogen source- or rapamycin-dependent changes in Aro80 phosphorylation monitored by protein mobility on SDS-PAGE. Therefore, it is not clear yet whether Aro80 is a direct target of TORC1 signaling pathway.

However, the induction of *ARO9* was significantly decreased in the deletion of *RTS1* gene (Fig 5.2). It was previously reported that *RTS1* gene encodes a regulatory subunit of PP2A. PP2A phosphatase is composed of a catalytic subunit, Pph21 or Pph22

Table 5.1 The candidate AGC kinases and non-AGC kinase proteins of Aro80 upstream signal.

AGC Kinase	Function
PKC1	Cell wall remodeling during growth
YPK1/2	YPK1 : Receptor-mediated endocytosis
	YPK2 : Optimal cell wall integrity
SCH9	The transcriptional activation of osmostress-responsive genes, G1 progression, cAPK activity and nitrogen activation of the FGM pathway
TPK1/2/3	Ras-cAMP signaling pathway
CBK1	Cell morphogenesis pathways
RIM15	The signal transduction during cell proliferation in response to nutrients
DBF2/DBF20	Cell cycle and morphogenesis
PKH1/2/3	The sphingolipid-mediated signaling pathway that controls endocytosis
IPL1	Aurora kinase subunit of the conserved chromosomal passenger complex
BUB1	The checkpoint regulation
KIN82	The regulation of phospholipid asymmetry
YNR047W	Regulation of the putative phospholipid translocases
YBR028C	Putative protein kinase, possible substrate of cAMP-dependent protein kinase (PKA)
YKL171W(NNK1)	Putative protein kinase, implicated in proteasome function
Other kinases and phosphatases	**Function**
KSP1	Filamentous growth
SKY1	mRNA metabolism and cation homeostasis
TAP42-PPH21/22	TORC1 downstream phosphatase
PPH21/22-TPD3-CDC55	TORC1 downstream phosphatase
PPH21/22-TPD3-RTS1	TORC1 downstream phosphatase

Table 5.2 Stress response pathway.

Pathway	Cellular cue	Major outputs	Intersection point(s) with TOR signaling
Calcineurin	Ca2+	Negative regulation of STRE genes, ion homeostasis	Slm1, Slm2, Fpr1
GCN2	Amino acid deprivation	Upregulation of amino acid anabolism and scavenging	Gcn2, eIF2α
SNF1	Glucose depletion	Activation of alternative carbon source utilization	Snf1, Gln3
Nitrogen discrimination	Quality of N source	Activation of non-preferred nitrogen source utilization	Ure2, Gln3, Gat1
Retrograde	Mitochondrial dysfunction	Replenishing of TCA cycle components	Lst8, Mks1, Rtg1, Rtg2,Rtg3
PKC	Osmotic homeostasis	Cell wall integrity	Rom2
PKA	Fermentable carbon source	Promotion of ribosome biogenesis, repression of stress response and G0 entrance	Fhl1, Ifh1, Sfp1, Maf1, Yak1, Msn2, Msn4, Rim15
PHG (diploid specific)	Poor N source	Foraging behavior-filamentous gr	Snf1, Mep2
Autophagy	N starvation	Autophagy	Atg1, Atg13

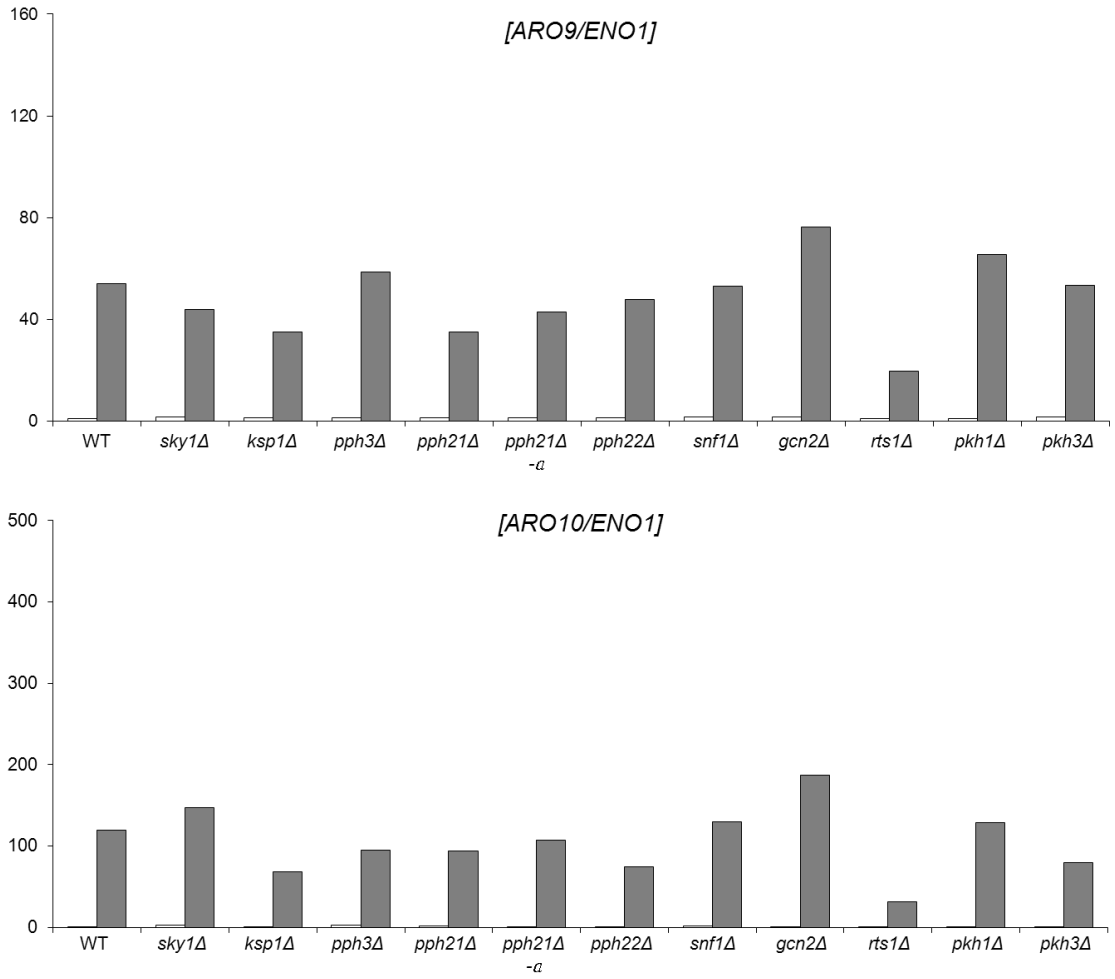


Figure 5.2 Screening of Aro80 upstream signal transduction pathway.

The effect of various candidates on the ability for expression of *ARO9* (A) and *ARO10* (B) under the condition of rapamycin treatment. WT, various deletion mutants were grown in YPD medium until early exponential phase and then treated with 0.2 μg/ml rapamycin (Rap) for 1 h. mRNA levels of *ARO9* and *ARO10* were determined by qRT-PCR normalized with *ENO1*.

complexes with a scaffold subunit, Tpd3, and one of two regulatory specificity subunits, Cdc55 or Rts1. The catalytic activity of Pph21/22 was found to maintain only in the presence of Rts1 and Cdc55. It was well-known that DNA binding and nuclear localization of GATA factors were regulated by PP2A. Therefore, this result suggests that PP2A might involve the expression of *ARO9* and *ARO10* via regulation of GATA factors.

5.5. Expression of Aro80 target genes depends on Pph21/22 phosphatase complex upon rapamycin treatment

PP2A and Sit4 phosphatases act downstream of TORC1 pathway to control Gat1 and Gln3 at the level of cellular localization and DNA binding [81]. Therefore, the roles for Sit4 and PP2A in rapamycin-dependent induction of Aro80 target genes were examined. Although rapamycin-dependent *DAL5* induction was reduced in *sit4Δ* as previously reported [81], induction of Aro80 targets genes was not much affected by the lack of *SIT4* (Fig 5.3 A). On the other hand, a double deletion mutant of two PP2A catalytic subunit genes, *PPH21* and *PPH22,* showed reduced rapamycin-induced expression of *ARO9* and *ARO10* as well as *DAL5* (Fig 5.3 A). Furthermore, deletion of other components of PP2A complex; *TPD3*, *CDC55*, and *RTS1*, also conveyed defects in the rapamycin-dependent induction of *ARO9* and *ARO10*. In agreement with the central role for Tpd3 in PP2A complex formation, *tpd3Δ* showed similar levels of defects in gene induction to those of *pph21Δpph22Δ* (Fig 5.3 B). The rapamycin-dependent induction levels of *ARO9* and *ARO10* were lower in a double deletion mutant *cdc55Δrts1Δ* than each single deletion mutant (Fig 5.3 B), indicating that both regulatory subunits are involved in the regulation of Aro80 target genes.

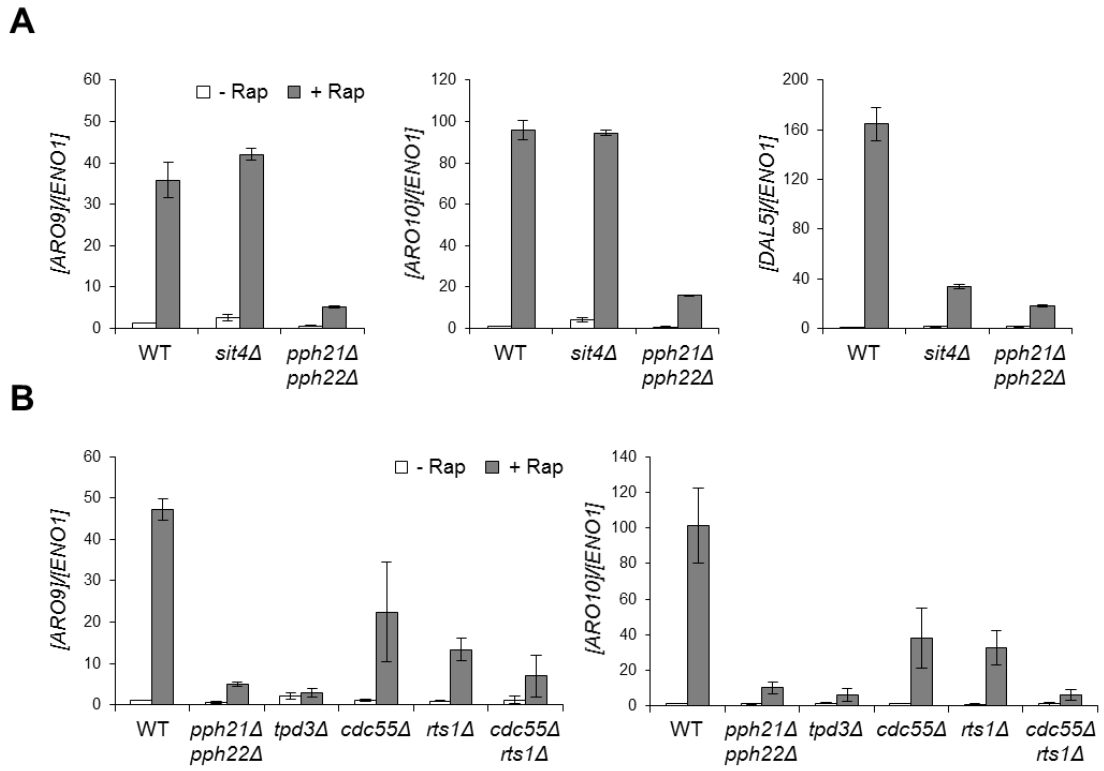


Figure 5.3 Expression of Aro80 target genes depends on Pph21/22 phosphatase complex upon rapamycin treatment.

A. Requirement of Pph21/22 phosphatase for rapamycin-dependent induction of *ARO9* and *ARO10*. BY4741 wild type, *sit4Δ* and *pph21Δ pph22Δ* cells were grown in YPD medium until early exponential phase and then treated 0.2 μg/ml rapamycin for 1 h. *ARO9*, *ARO10* and *DAL5* mRNA expression were quantified by qRT-PCR normalized with *ENO1*. Each value represents the average of three independent experiments.

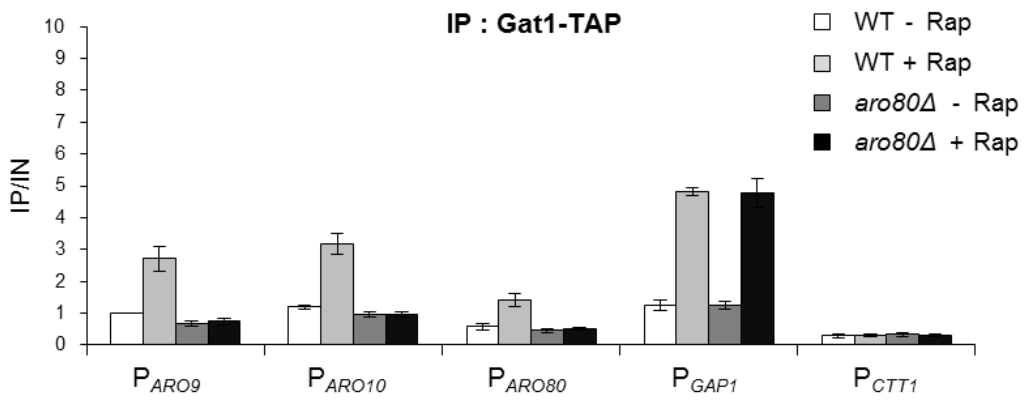
B. Effects of the components of Pph21/22 phosphatase complex on rapamycin-induction of *ARO9* and *ARO10*. Rapamycin-induced expression levels of *ARO9* and *ARO10* were detected by qRT-PCR in the indicated strains.

5.6. Gat1 and Gln3 bind to the Aro80 target promoters in an Aro80 dependent-manner

Rapamycin-dependent induction of *ARO9* and *ARO10* was almost abolished in *aro80Δ* even in the presence of Gln3 and Gat1. This result can be explained if Aro80 is a sole regulator involved in the rapamycin-dependent induction of its target genes, and the effects of GATA factors are indirect through regulating intracellular inducer levels as suggested previously [2]. On the other hand, if GATA factors directly regulate Aro80 targets genes, Aro80 might be required for the recruitment of GATA factors to the promoters.

To elucidate the regulatory mechanisms, this study investigated binding of Gat1 and Gln3 to the Aro80 target promoters by ChIP analyses. Gat1-TAP and Gln3-TAP showed increased binding to the *ARO9*, *ARO10*, and *ARO80* promoters upon rapamycin treatment, demonstrating a direct role for Gat1 and Gln3 in their expression (Fig 5.4 A, B). However, the rapamycin-dependent recruitment of Gat1 to theses promoters was completely abolished in *aro80Δ* (Fig 5.4 A). Compared with Gat1, Gln3 showed weaker Aro80 requirement for DNA binding, exhibiting about 50% reduction in binding in the absence of Aro80 (Fig 5.4 B). Interestingly, Aro80, which cannot activate *ARO80* in response to aromatic amino acids, seems to be functional in the recruitment of GATA factors to the *ARO80* promoter, suggesting that Aro80 transactivation activity is not absolutely required for the recruitment of GATA factors. However, rapamycin-dependent recruitment of Gat1 and Gln3 to the control *GAP1* promoter was not affected by *ARO80* deletion (Fig 5.4 A, B). These results suggest that the critical role for Aro80 in the rapamycin-dependent induction of its target genes

A


IP : Gat1-TAP
WT - Rap
WT + Rap
aro80Δ - Rap
aro80Δ + Rap
IP/IN
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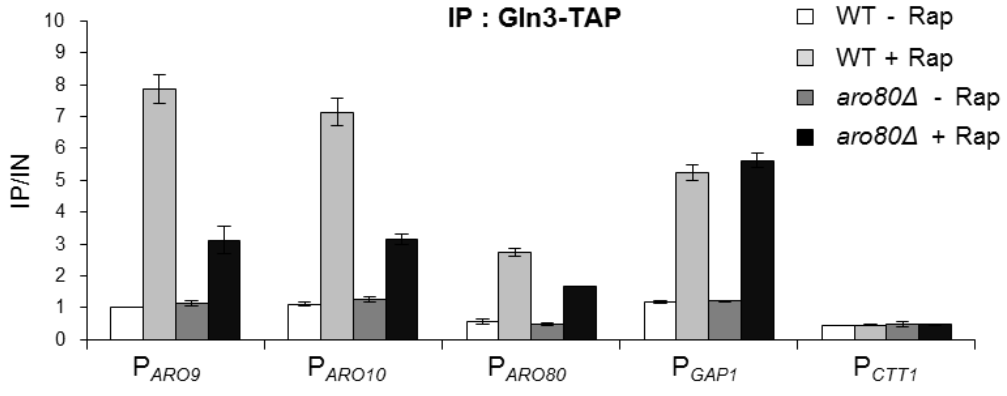
P GAP1
P CTT1

B


IP : Gln3-TAP
WT - Rap
WT + Rap
aro80Δ - Rap
aro80Δ + Rap
IP/IN
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P ARO9
P ARO10
P ARO80
P GAP1
P CTT1

Figure 5.4 Gat1 and Gln3 bind to the Aro80 target promoters in an Aro80 dependent-manner.

A. Effect of *ARO80* deletion on Gat1-TAP binding to the Aro80 target promoters. Gat1 binding to the *ARO9*, *ARO10* and *ARO80* promoters was determined by ChIP. JHY206 (WT Gat1-TAP) and JHY207 (*aro80*Δ Gat1-TAP) cells were grown in YPD medium until early exponential phase and then treated with 0.2 μg/ml rapamycin for 1 h. The amounts of Gat1-TAP bound to the Aro80 target promoters and *GAP1* promoter were determined by ChIP. The *CTT1* promoter was used as a negative control. Results are expressed as relative IP/IN values normalized to that of P_{ARO9} in untreated wild type.

B. Effect of *ARO80* deletion on Gln3-TAP binding to the Aro80 target promoters. Rapamycin-dependent binding of Gln3-TAP to the Aro80 target promoters and *GAP1* promoter was detected by ChIP in JHY208 (WT Gln3-TAP) and JHY209 (*aro80*Δ Gln3-TAP) cells grown in YPD medium. Results are expressed as relative IP/IN values normalized to that of P_{ARO9} in untreated wild type.

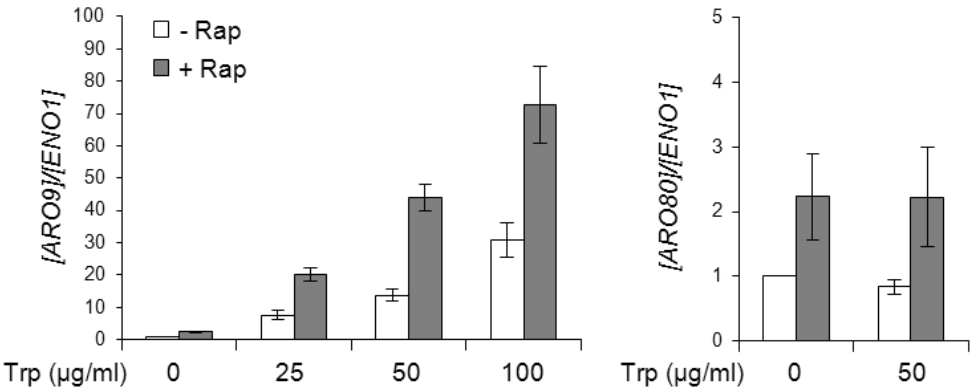
might be due to the Aro80-dependent binding of Gat1 and Gln3 to the promoters. In addition, as discussed above, activation of Aro80 by increased inducer levels might also contribute to the effect of Aro80 on the rapamycin-dependent induction of *ARO9* and *ARO10*. Although

5.7. Aro80 activity is not required for Gat1 and Gln3 binding to the Aro80 target promoters

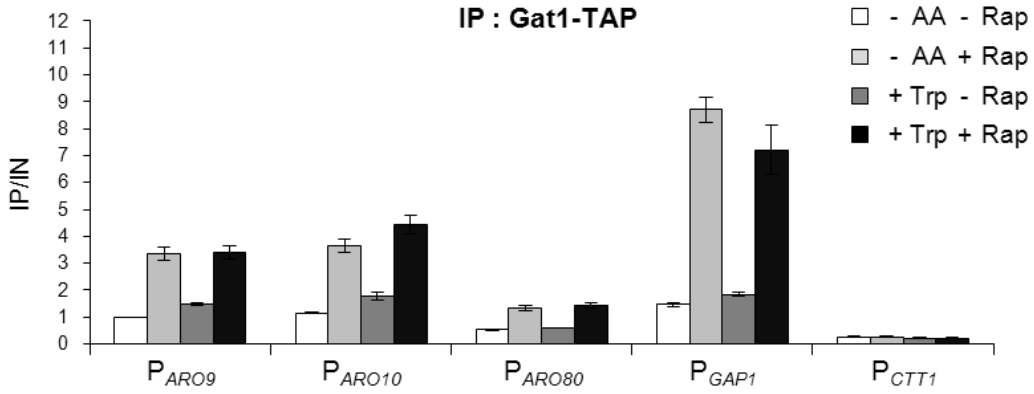
Although activity of Aro80 might not be necessary for the recruitment of Gat1 and Gln3 to the Aro80 target promoters, it was examined whether Aro80 activity can play an additional regulatory role for the binding of GATA factors to *ARO9* and *ARO10* promoters. Cells grown in minimal ammonia medium containing increasing amounts of tryptophan showed concentration-dependent increase in basal *ARO9* expression levels, exhibiting up to 30-fold induction in the presence of 100 μg/ml tryptophan compared with cells grown in the absence of amino acids (Fig 5.5 A). Treatment of rapamycin elicited about 2.3-to 3.2-fold induction of *ARO9* expression irrespective of tryptophan concentration (Fig 5.5 A), suggesting that activation by GATA factors might not be affected by Aro80 activity. Although rapamycin-dependent induction was also observed in the absence of amino acids, the induced expression level was negligible because of the very low basal expression level of *ARO9*. The rapamycin-dependent induction fold of *ARO9* was much lower in minimal medium than YPD medium.

This study also examined rapamycin-dependent binding of GATA factors to the Aro80 target promoters in the absence or presence of tryptophan. Rapamycin-induced binding of Gat1-TAP and Gln3-TAP to the *ARO9*, *ARO10*, and *ARO80* promoters was observed in a tryptophan-independent manner (Fig 5.5 B, C), demonstrating that Aro80 activity might not affect the recruitment of Gat1 and Gln3 to the promoters. Therefore, irrespective of its activity, Aro80 binding to the promoters might be enough to allow binding of GATA factors.

A


- Rap
+ Rap
[ARO9]/[ENO1]
[ARO80]/[ENO1]
Trp (µg/ml) 0 25 50 100
Trp (µg/ml) 0 50

B


IP : Gat1-TAP
- AA - Rap
- AA + Rap
+ Trp - Rap
+ Trp + Rap
IP/IN
P ARO9
P ARO10
P ARO80
P GAP1
P CTT1

C

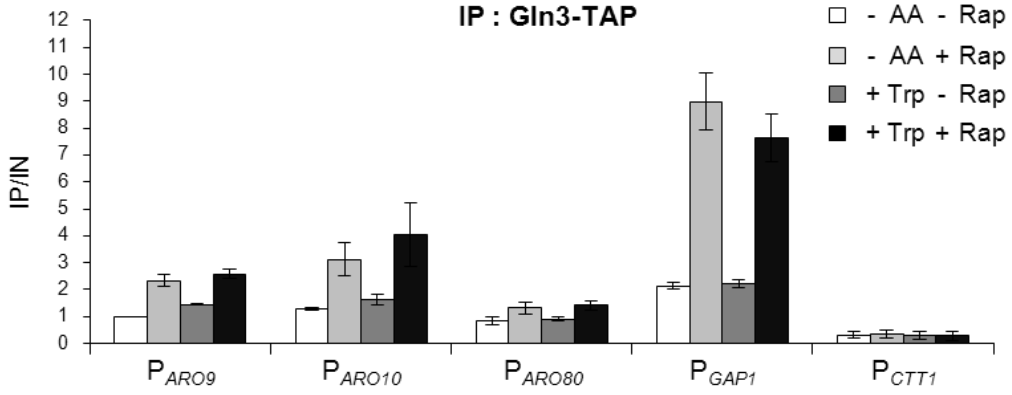

IP : Gln3-TAP
- AA - Rap
- AA + Rap
+ Trp - Rap
+ Trp + Rap
IP/IN
P ARO9
P ARO10
P ARO80
P GAP1
P CTT1

Figure 5.5 Aro80 activity is not required for Gat1 and Gln3 binding to the Aro80 target promoters.

A. Effect of tryptophan on *ARO9* and *ARO80* expression in the absence or presence of rapamycin. BY4741 cells were grown in minimal medium lacking amino acids or containing the indicated amounts of tryptophan until early exponential phase and then treated with rapamycin for 1 h. *ARO9* and *ARO80* mRNA levels were analysed by qRT-PCR normalized with *ENO1*.

B. Tryptophan-independent recruitment of Gat1-TAP to the Aro80 target promoters upon rapamycin treatment. JHY206 (WT Gat1-TAP) cells were grown in minimal medium lacking amino acids (−AA) or containing 50 μg/ml tryptophan (+Trp). Gat1-TAP binding to the promoters of Aro80 target genes and *GAP1* was monitored by ChIP before and after rapamycin treatment. The *CTT1* promoter was used as a negative control. Results are expressed as relative IP/IN values normalized to that of P*ARO9* in untreated control (− AA − Rap).

C. Tryptophan-independent recruitment of Gln3-TAP to the Aro80 target promoters upon rapamycin treatment. JHY208 (WT Gln3-TAP) cells were grown in minimal medium lacking amino acids (− AA) or containing 50 μg/ml tryptophan (+ Trp). Gln3-TAP binding to the promoters of Aro80 target genes and *GAP1* was monitored by ChIP before and after rapamycin treatment. Results are expressed as relative IP/IN values normalized to that of P*ARO9* in untreated control (− AA − Rap).

In addition, it was noticed that the fold increases in the rapamycin-induced Gln3 binding to the *ARO9*, *ARO10*, and *ARO80* promoters were lower in minimal medium than those in YPD medium approximately by 3-fold (Fig 5.5 C), whereas Gat1 binding was largely unaffected by the media types (Fig 5.5 B). Although further studies are required to understand the media-dependent differential regulation of Gat1 and Gln3, the higher Gln3 binding in YPD medium might contribute to the higher rapamycin-dependent induction of Aro80 target genes in YPD medium compared with minimal medium. In addition, indirect inducer-mediated Aro80 activation might be more prominent in rich YPD medium than minimal medium upon rapamycin treatment.

5.8. GATA factors are not required for Aro80 binding to the promoters

Next, it was examined whether GATA factors can affect Aro80 binding to the promoters in a reverse way. To this end, the binding affinity of Aro80-HA to Aro80 target promoters in wild type and *gln3Δgat1Δ* were compared. For this experiment, Aro80-HA was constitutively expressed from *ADH1* promoter in the *aro80Δ* background, excluding the effect of the transcriptional induction of *ARO80* upon rapamycin treatment. Even in the absence of the transcriptional regulation of *ARO80*, *ARO9* was induced by rapamycin in a similar manner similar to that observed in wild type (Fig 5.6 A). This result suggests that GATA factor-dependent *ARO80* induction might not play an essential role in the rapamycin-dependent induction of *ARO9*, although it might have some regulatory roles in fine-tuning of the overall regulatory network.

ChIP analyses showed lower binding affinity of Aro80-HA to the *ARO80* promoters than to the *ARO9* and *ARO10* promoters (Fig 5.6 B), which might be related to the fact that the *ARO80* promoter contains only two CCG repeats, while the *ARO9* and *ARO10* promoters have four CCG repeats. Although it is weak, Aro80 binding to its own promoter might be significant considering the fact that Aro80 is required for the recruitment of GATA factors to the *ARO80* promoter. Aro80 binding to these promoters was not noticeably changed by rapamycin treatment in both wild type and *gln3Δgat1Δ* (Fig 5.7 B). Moreover, Aro80 binding levels to the target promoters were not markedly different between wild type and *gln3Δgat1Δ*. Therefore, while constitutively bound Aro80 can facilitate the binding of GATA factors upon rapamycin treatment, GATA factors seem to not play any positive role for the binding of Aro80.

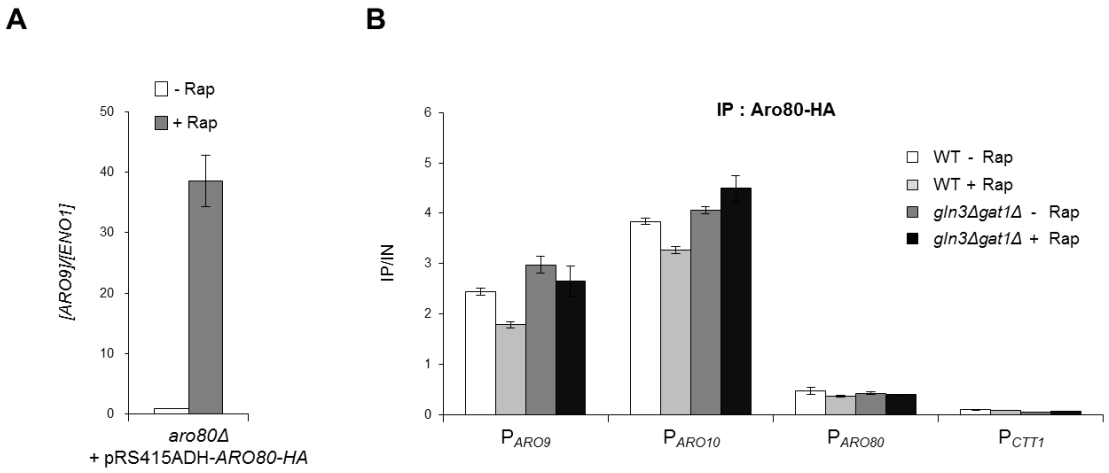


Figure 5.6 GATA factors are not required for Aro80 binding to the promoters.

A. Aro80-HA is functional in rapamycin-dependent induction of *ARO9*. BY4741 *aro80*Δ cells expressing pRS415ADH-Aro80-HA were grown in minimal medium until early log phase and then the medium was changed to YPD. The cells were treated with 0.2 μg/ml rapamycin for 1 h, and *ARO9* mRNA levels were determined by qRT-PCR normalized to *ENO1*.

B. Effects of GATA factors on binding of Aro80-HA to the Aro80 target promoters. WT and *gln3*Δ*gat1*Δ cells expressing Aro80-HA were grown in minimal medium until early exponential phase and then treated with 0.2 μg/ml rapamycin for 1 h. The amounts of Aro80-HA bound to the Aro80 target promoters were determined by ChIP. The *CTT1* promoter was used as a negative control.

5.9. Poor nitrogen sources activate transcription of Aro80 target genes

The expression levels of *ARO9* and *ARO10* were also investigated in the presence of different nitrogen sources (Fig 5.7). The results showed a typical pattern of NCR, exhibiting higher expression levels of *ARO9* and *ARO10* in the presence of poor nitrogen sources such as proline and urea compared with good nitrogen sources like glutamine and ammonia (Fig 5.7 A). Addition of tryptophan increased the overall expression levels while exhibiting the same nitrogen source-dependent expression pattern (Fig 5.7 B). Therefore, although differential regulation of Gat1 and Gln3 in response to rapamycin and poor nitrogen source might exert different contribution of each GATA factor to the regulation of Aro80 target genes, the interplay between Aro80 and GATA factors suggested in our model for rapamycin treatment might also be conserved in response to different nitrogen sources.

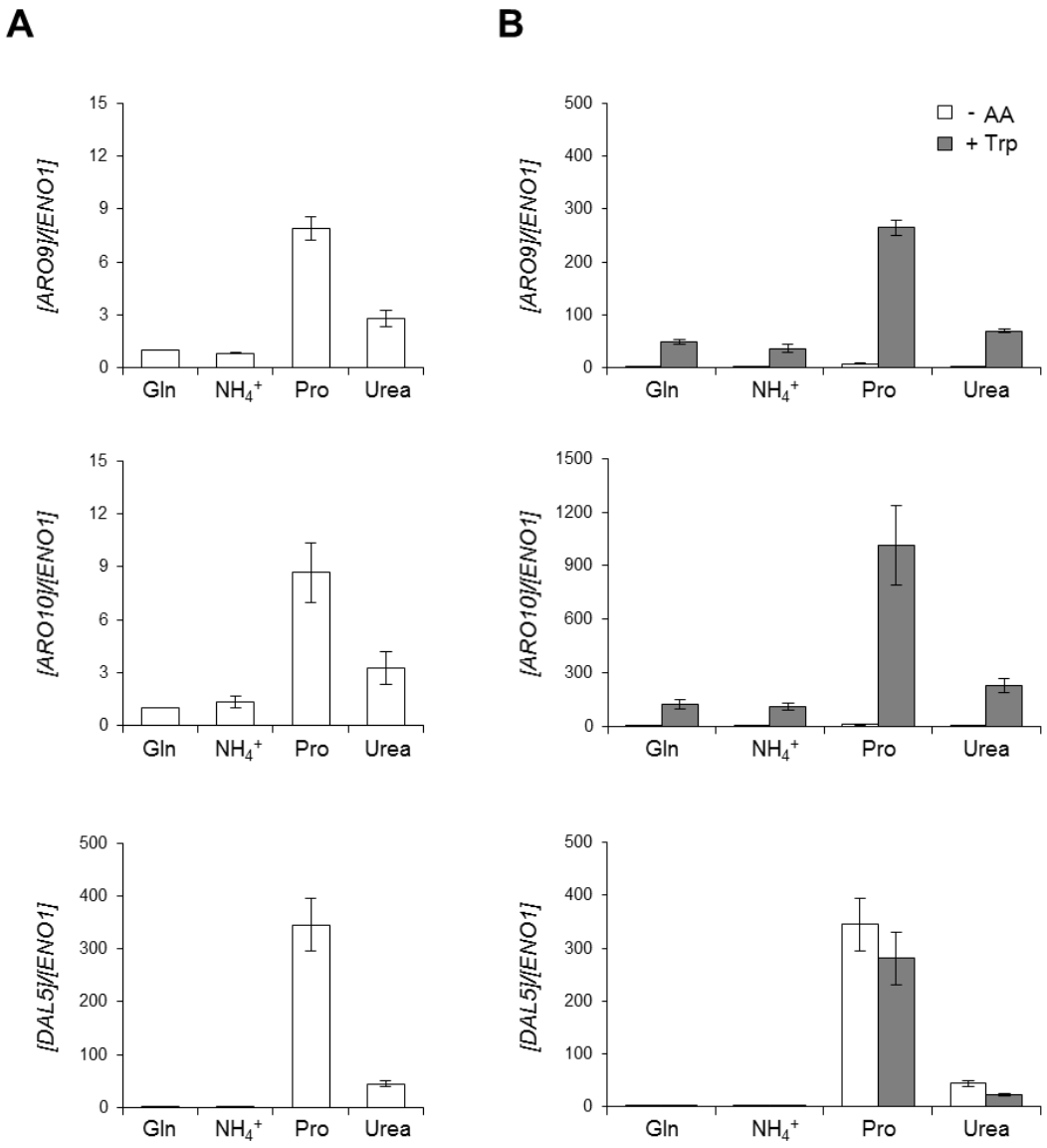

A
B
- AA
+ Trp
[ARO9]/[ENO1]
[ARO10]/[ENO1]
[DAL5]/[ENO1]
Gln
NH4+
Pro
Urea

Figure 5.7 Poor nitrogen sources activate transcription of Aro80 target genes.

A. Effect of poor nitrogen sources on transcription of Aro80 target genes. BY4741 cells were grown to early log phase in minimal medium containing 0.1% glutamine, 0.1% ammonium sulfate, 0.1% proline, or 0.1% urea as sole nitrogen source. *ARO9*, *ARO10* and *DAL5* mRNA expression levels were quantified by qRT-PCR normalized with *ENO1*.

B. Effect of poor nitrogen sources on transcription of Aro80 target genes in the presence of tryptophan. Yeast cells were grown to early log phase in minimal medium containing the indicated nitrogen sources and 100 μg/ml tryptophan (+ Trp). *ARO9*, *ARO10* and *DAL5* mRNA expression were quantified by qRT-PCR normalized with *ENO1*. The data showing mRNA expression levels in the absence of amino acids (− AA) are the same results shown in (A).

5.10. Gzf3 and Dal80 negative GATA factors are not involved in rapamycin-dependent induction of Aro80 target genes

It is well known that the gene expressions of NCR genes are up-regulated by Gln3 and Gat1 positive GATA factors [1]. In addition the regulation of the Gln3 and Gat1 activators, the expression of NCR genes is regulated by the GATA repressors Dal80 and Gzf3 [81]. Also, it was previously reported that negative GATA factors bind to GATAA-containing promoter *in vitro*. The binding of Dal80 requires two GATAAG sequences [82]. On the contrary, Gzf3 can bind a single GATAAG sequence. Because the zinc finger DNA binding domain of Dal80 and Gzf3 are greatly homologous with those of other GATA activators, this character was expected and that they repress by competing with Gat1 or Gln3 for the GATA sites. However the two negative GATA factors play very different roles in the regulation of NCR genes. Dal80 regulates the expression of NCR genes only in the presence of non-preferred nitrogen source. However, Gzf3 regulates the transcription of NCR genes under conditions of nitrogen repression.

In this chapter, it was shown that positive GATA factors are involved in activation of *ARO9* and *ARO10* genes. Thus, the roles for negative GATA factors in rapamycin-dependent induction of Aro80 target genes were examined. From results, it was found that the transcription of *ARO9* and *ARO10* genes was not affected by deletion of *dal80Δ* or *gzf3Δ* stains upon rapamycin treatment (Fig 5.8). The expression of *DAL5* significantly was increased in *gzf3Δ* strain upon rapamycin treatment as previously reported (Fig 5.8). Next, it was investigated whether negative GATA factors are involved in the expression of *ARO9* and *ARO10* under poor nitrogen source like

proline. *ARO9* expression was detected in glutamine or proline cultured wild type, *dal80Δ* or *gzf3Δ* stains. In proline-grown cells, high level *DAL5* expression observed in *dal80Δ* or *gzf3*Δ as previously reported (Fig 5.9). However, the expression of *ARO9* and *ARO10* was not changed by deletion of *dal80Δ* or *gzf3Δ* under proline media (Fig 5.9). Results are from experiments with varying conditions and it was concluded that negative GATA factors are not involved in the regulation of Aro80 target genes under condition of rapamycin treatment or poor nitrogen source.

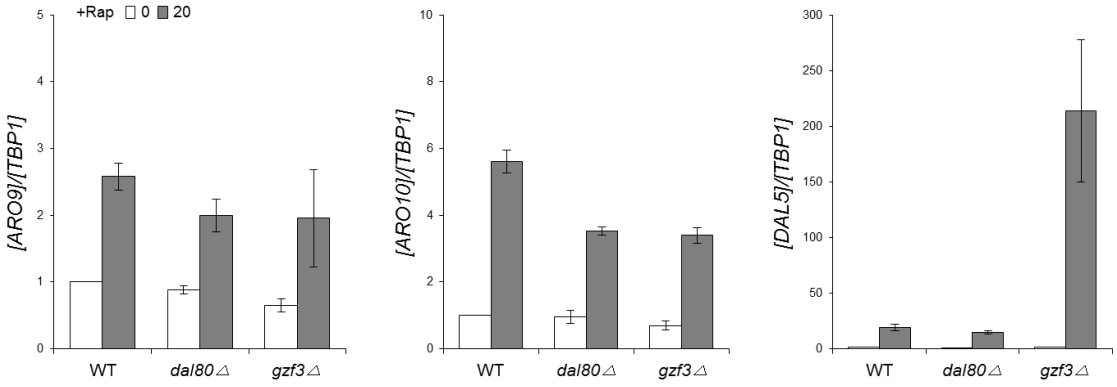


Figure 5.8 Effect of negative GATA factors on expression of *ARO9* and *ARO10* genes under rapamycin treatment.

Effects of Dal80 and Gzf3 on rapamycin-dependent induction of Aro80 target genes. WT, *dal80Δ* and *gzf3*Δ cells were cultured in YPD medium until early exponential phase and then treated with 0.2 μg/ml rapamycin (Rap) for 20 min. mRNA levels of *ARO9*, *ARO10* and *DAL5* were determined by qRT-PCR normalized with *TBP1*. Each value represents the average of three independent experiments.

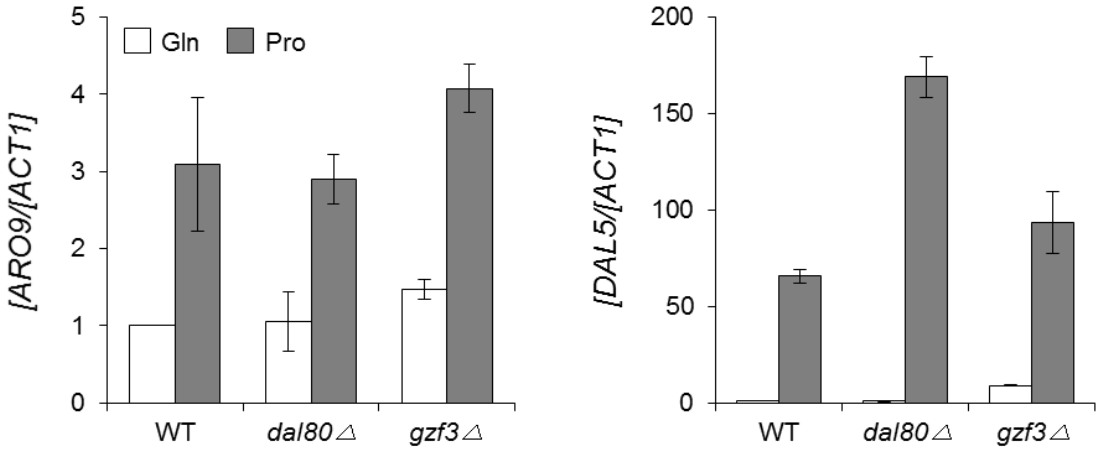


Figure 5.9 Effect of negative GATA factors on expression of *ARO9* and *ARO10* genes under proline media.

WT, *dal80Δ* and *gzf3*Δ cells were grown to early log phase in minimal medium containing 0.1% proline as sole nitrogen source. *ARO9* and *DAL5* mRNA expression levels were quantified by qRT-PCR normalized with *ACT1*.

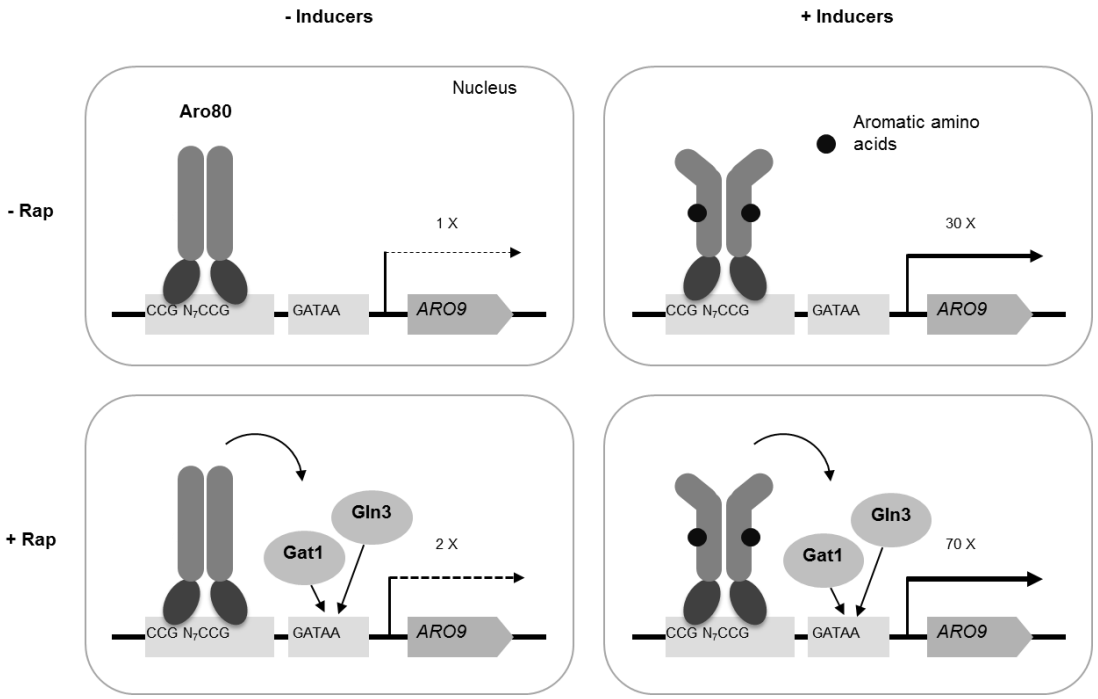


Figure 5.10 Model for transcriptional regulation of *ARO9* by Aro80 and GATA activators.

Aro80 is bound to the *ARO9* promoter even in the absence of inducers (aromatic amino acids) and activated by inducers. Upon rapamycin treatment, Gat1 and Gln3 are recruited to the promoter in an Aro80-dependent manner irrespective of Aro80 activity. The indicated relative *ARO9* expression levels are based on the data shown in Fig 5.8 A, which were obtained from cells grown in minimal medium lacking amino acids (− inducers) and containing 100 μg/ml tryptophan (+ inducers).

5.11. Conclusion

In this chapter, it has been shown that transcription of genes involved in catabolism of aromatic amino acids is regulated by interplay between Aro80 and GATA activators. Aro80 is required for GATA factor binding to the Aro80 target promoters. Although many of NCR genes are regulated both positive GATA factors and negative GATA factors, negative GATA factors do not affect the expression of *ARO9* and *ARO10*. In addition, positive GATA factors might indirectly influence Aro80 activity through transcriptional regulation of amino acid permeases importing Aro80 inducers. Therefore, GATA factors might play both direct and indirect roles in transcriptional regulation of Aro80 target genes.

Chapter 6.

Heat shock induces the transcription of *ARO9* and *ARO10* by increasing amino acid influx

6.1. 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). 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[1]. 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[41].

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 [5]. Aro9 transaminase and Aro10 decarboxylase are involved in the first two steps of the aromatic amino acid catabolism via Ehrlich pathway [5]. Transcription of the *ARO9* and *ARO10* genes is regulated both by Aro80, a pathway-specific regulator, and by GATA activators. 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. In addition, it 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 [83].

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

6.2. Aro80 is activated by heat shock

Transcription of the *ARO9* and *ARO10* genes are regulated by aromatic amino acids and nitrogen quality. This study investigated whether other environmental conditions can affect the expression of these genes. This study 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 6.1). Heat shock induction of *ARO9* and *ARO10* in a rich YPD medium was completely abolished in *aro80Δ* strain, suggesting that Aro80 can be activated by heat shock (Fig 6.1 A). Heat shock induction of *HSP26*, a target of Msn2/4 and Hsf1 transcription factors [84] , was not affected by the lack of *ARO80*, confirming the specific role for Aro80 in heat shock induction of its target genes (Fig 6.1 B). Gln3 and Gat1 directly activate the expression of *ARO9* and *ARO10* in the presence of poor source or rapamycin. However, heat shock-dependent induction level 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 6.1 C).

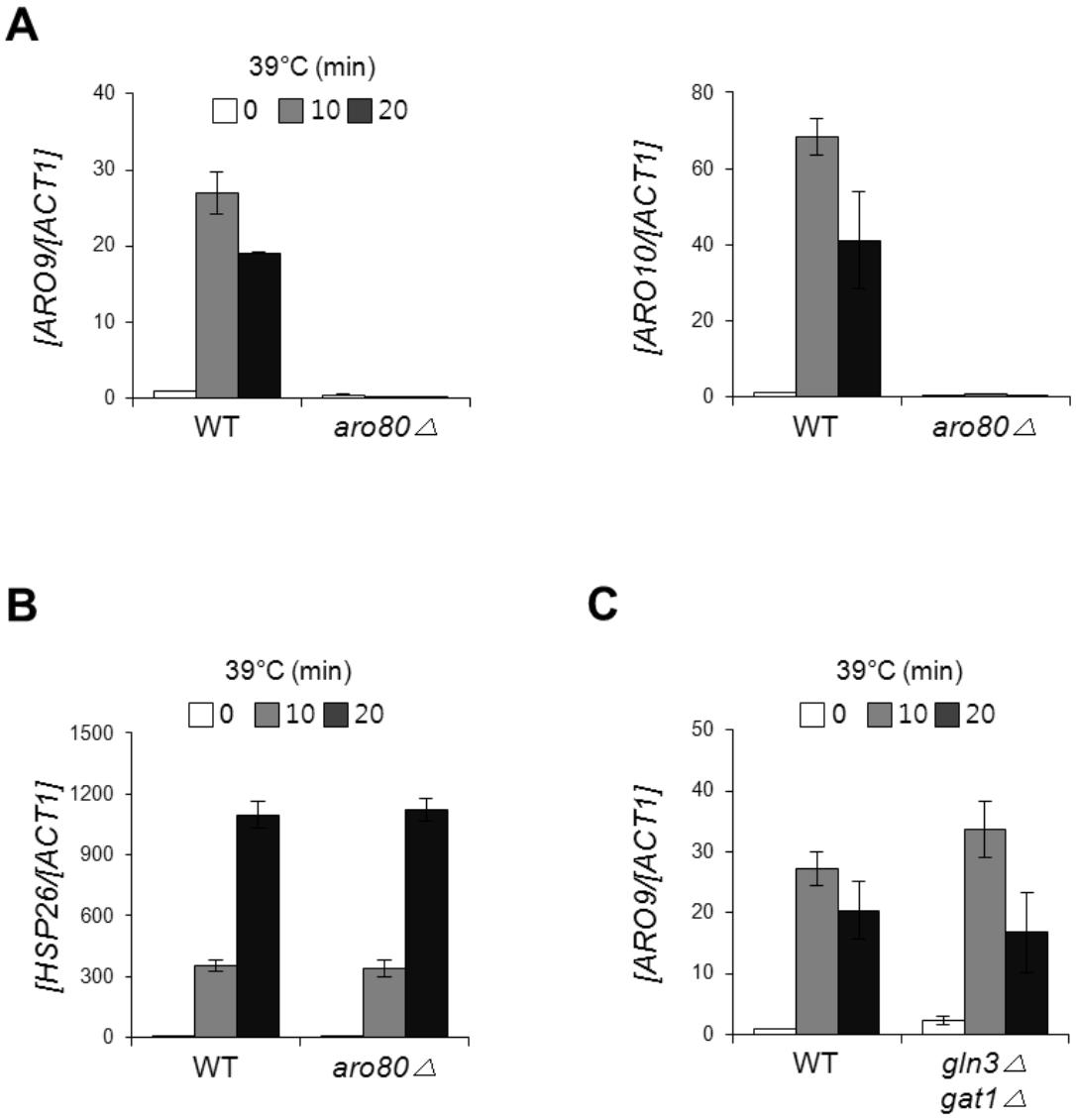

A
39°C (min)
0
10
20
[ARO9/[ACT1]
40
30
20
10
0
WT
aro80△
[ARO10/[ACT1]
80
60
40
20
0
WT
aro80△
B
39°C (min)
0
10
20
[HSP26/[ACT1]
1500
1200
900
600
300
0
WT
aro80△
C
39°C (min)
0
10
20
[ARO9/[ACT1]
50
40
30
20
10
0
WT
gln3△
gat1△

Figure 6.1 Expression of *ARO9* and *ARO10* is activated by heat shock in an Aro80-dependent manner.

A. Aro80-dependent induction of *ARO9* and *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* and *ARO10* were analyzed by qRT-PCR normalized with *ACT1*. Each value represents the average of three independent experiments, and error bars indicate standard deviations.

B. Expression level of *HSP26* 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 *HSP26* were analyzed by qRT-PCR normalized with *ACT1*. Each value represents the average of three independent experiments, and error bars indicate standard deviations.

C. Effect of Gat1 and Gln3 on the heat shock induction of *ARO9*. *ARO9* mRNA expression levels were analyzed by qRT-PCR in WT and *gln3Δ gat1Δ* before and after heat shock.

6.3. Heat shock activation of genes involved in amino acid catabolism

Several NCR genes such as *PUT1*, *PUT2*, *UGA4*, *CAR1* and *CAR2* are regulated by pathway-specific transcription factor upon ligand treatment and general transcription factors under nitrogen starvation. Especially, transcription of these genes is rapidly and dramatically up-regulated in response to specific amino acid ligands, as well as in response to rapamycin which cause TORC1 inactivation.. Because there are similar properties between Aro80 target genes and general NCR genes, it was tested whether *PUT1* and *CAR1* genes are regulated by heat shock. Interestingly, results showed that expression levels of *PUT1* and *CAR1* were also increased by heat shock about 4 fold in an Aro80-independent manner (Fig 6.2).

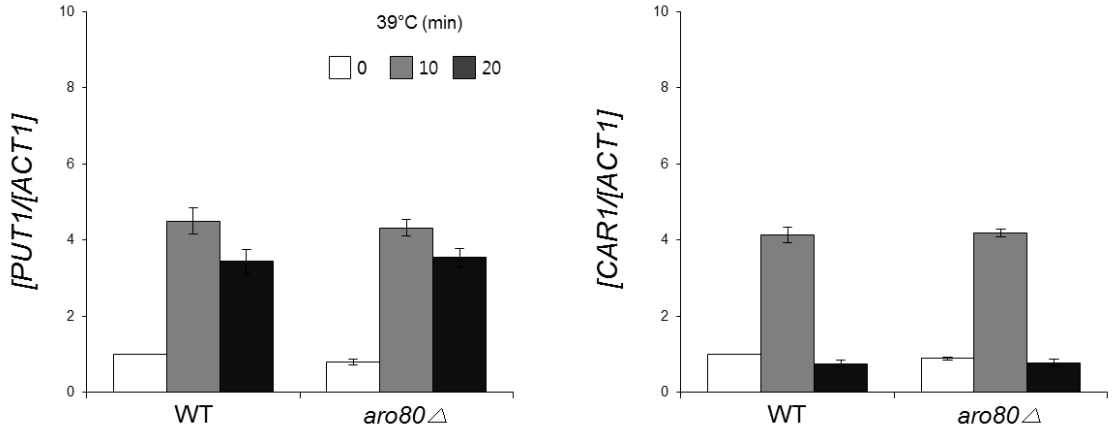


Figure 6.2 Expression of *PUT1* and *CAR1* is activated by heat shock in an Aro80-independent manner.

Aro80-independent induction of *PUT1* and C*AR1* 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 *PUT1* and C*AR1* were analyzed by qRT-PCR normalized with *ACT1*. Each value represents the average of three independent experiments, and error bars indicate standard deviations.

6.4. 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 (HOG1) signaling pathway are implicated in heat shock response in *S. cerevisiae*. Therefore, it was 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 decrease in GTP hydrolysis of Ras proteins, respectively. Heat shock induction of *CTT1*, a known target of Msn2/4 which is negatively regulated by PKA [85], was reduced in *pde2Δ* and *ira2Δ* mutants (Fig 6.3 A). However, expression of *ARO9* was not much affected by the deletion of *PDE2* or *IRA2*, suggesting that PKA pathway might not regulate Aro80 activation. This study also examined the possible involvement of Hog1 signaling pathway in heat shock induction of *ARO9*. However, deletion mutants of *SHO1* and *SSK1* involved in Hog1 signaling pathway [86] showed no defect in heat shock induction of *ARO9* (Fig 6.3 B). Furthermore, deletion of *WSC1* and *ROM2* in protein kinase C (PKC) signaling pathway [87] also showed normal induction of *ARO9* upon heat shock (Fig 6.3 C).

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

Since heat shock induction of *ARO9* in YPD medium was not affected by the known signaling pathways, this study hypothesized that heat shock activation of Aro80 might be involved in an increase in the availability of aromatic amino acids which act as inducers. It was also possible that increasing temperature might affect an increase in rate of reaction which up-regulate Aro80 transcription factor activity. Also increase in the intracellular amino acid levels can be achieved either by increase in amino acid uptake from the medium or by increase in intracellular free amino acids pool possibly by protein degradation or autophage. To elucidate the regulatory mechanism, it was 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 (100 μg/ml of each His, Leu, Met, Ura), both basal and heat-induced expression levels of *ARO9* and *ARO10* were lower than those in a minimal medium containing amino acids (Fig 6.4). *ARO9* and *ARO10* were induced 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 amino acids in the medium. The increase of Aro80 activity, protein degradation or autophage might contribute the slight increase of *ARO9* and *ARO10* expression in a minimal medium lacking amino acids (-AA).Therefore, increase in amino acids uptake rather than internal supply of free amino acids might be mainly responsible for Aro80 activation upon heat shock.

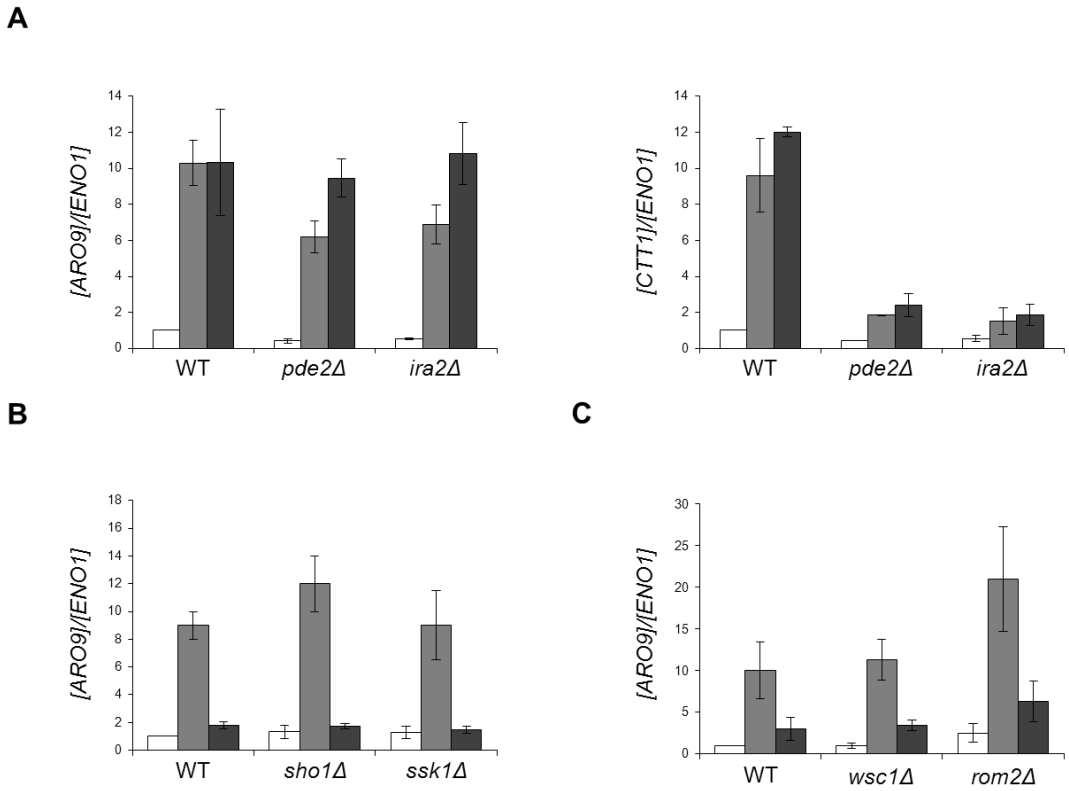


Figure 6.3 Effect of PKA, PKC and Hog1 signaling pathway on heat shock dependent induction of *ARO9* gene.

A. 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.

B. Effect of Hog1 pathway on the heat shock induction of *ARO9*. *ARO9* mRNA levels were analyzed by qRT-PCR in WT, *sho1Δ*, and *ssk1Δ* before and after heat shock.

C. Effect of PKC pathway on the heat shock induction of *ARO9*. *ARO9* mRNA levels were analyzed by qRT-PCR in WT, *wsc1Δ*, and *rom2Δ* before and after heat shock.

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

Next, it was investigated whether intracellular aromatic amino acid concentrations can be changed upon heat shock. Cells were grown in the presence or absence of amino acids at 30℃, and then heat shocked at 39℃ for 15 min. The intracellular amino acids levels were detected by mass spectrometry-based analysis. When cells were grown in a medium containing amino acids, intracellular Phe, Tyr and Trp concentrations were increased by 1.5-fold in average upon heat shock (Fig 6.5). On the other hand, for cells grown in the absence of amino acids, the concentrations of Phe and Tyr were not significantly changed before and after heat shock, and Trp concentration was below the detection limit. These results also support the notion that heat shock might induce increase in amino acids uptake from outside of the cells.

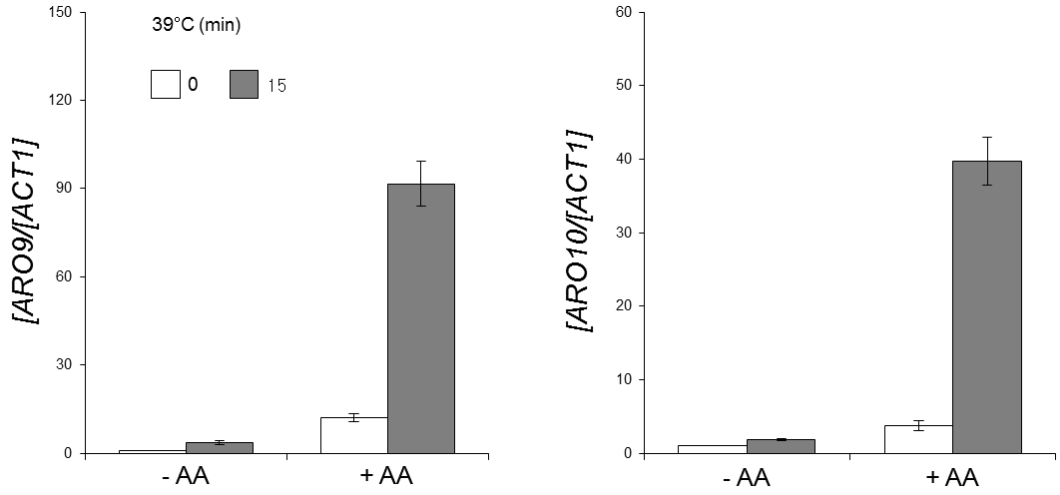


Figure 6.4 Effect of extracellular amino acids on the heat shock induction of *ARO9* and *ARO10.*

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*.

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

Heat-induced amino acids 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 [88,89], which can facilitate passive influx of amino acids. To elucidate the mechanism, the roles of plasma membrane amino acid permeases in the heat-induced expression of *ARO9* and *ARO10* were investigated. It has been known that Gap1, Agp1, Tat2, Bap2, and Bap3 permeases are involved in the transport of aromatic amino acids [90,91]. However, heat-induced expression levels of *ARO9* and *ARO10* were not significantly changed in the deletion mutants lacking each permease (Fig 6.6 A). Furthermore, a double deletion mutant *gap1Δagp1Δ*, which has been shown to have a defect in the Trp-dependent activation of *ARO9*-lacZ in a urea medium [2], also showed normal induction of *ARO9* and *ARO10* by heat shock (Fig 6.6 B). These results suggest that amino acid permeases are not involved in the heat-induced amino acids uptake. Although this study cannot rule out the possibility that other amino acid permeases are responsible for the aromatic amino acids uptake during heat shock, increase in membrane fluidity might be the major reason for the heat-induced amino acid influx, which in turn activates Aro80, leading to the induction of *ARO9* and *ARO10* genes (Fig 6.6).

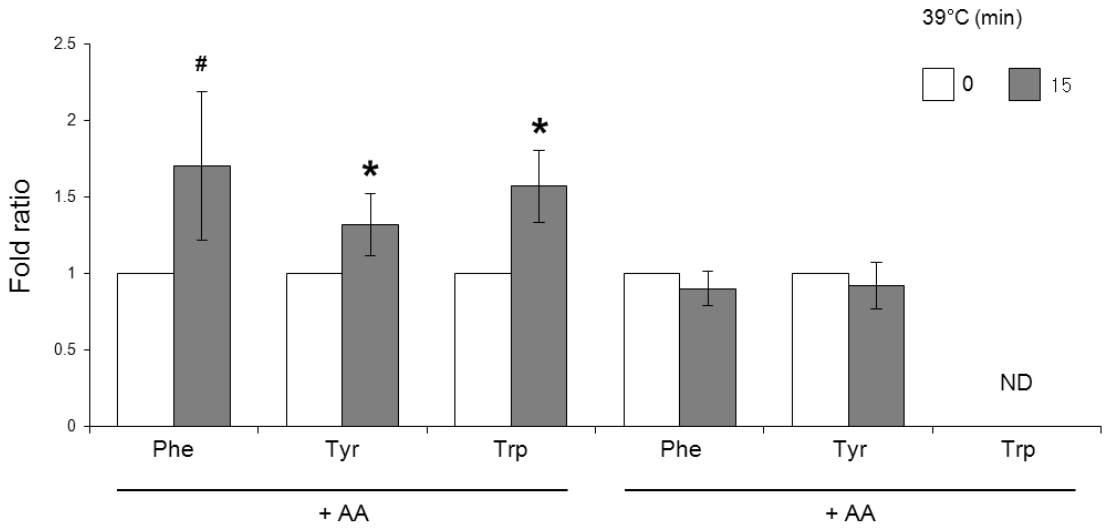
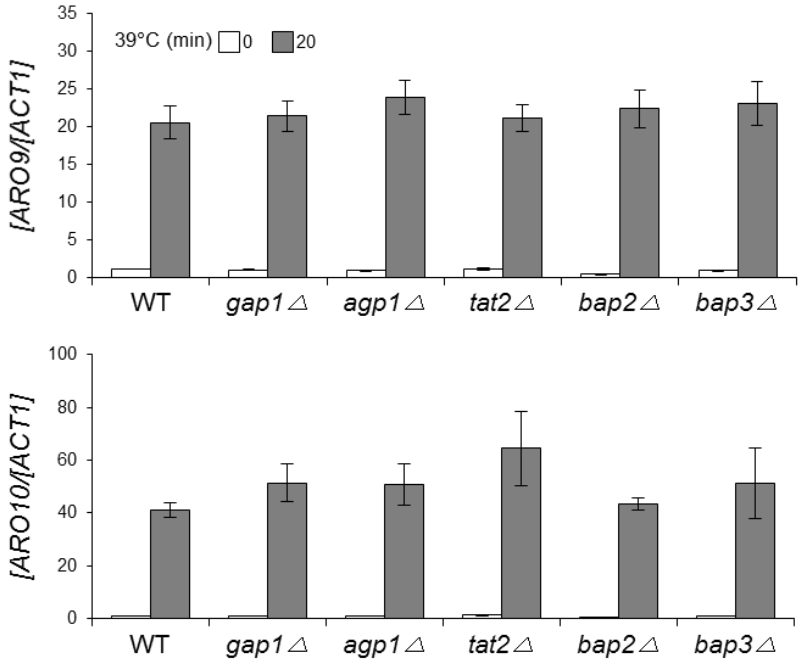


Figure 6.5 Increase in intracellular aromatic amino acid concentrations upon heat shock.

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 (#, $P < 0.1$; *, $P < 0.05$).

A


39°C (min) 0 20
[ARO9/[ACT1]
35
30
25
20
15
10
5
0
WT
gap1⊿
agp1⊿
tat2⊿
bap2⊿
bap3⊿
[ARO10/[ACT1]
100
80
60
40
20
0
WT
gap1⊿
agp1⊿
tat2⊿
bap2⊿
bap3⊿

B

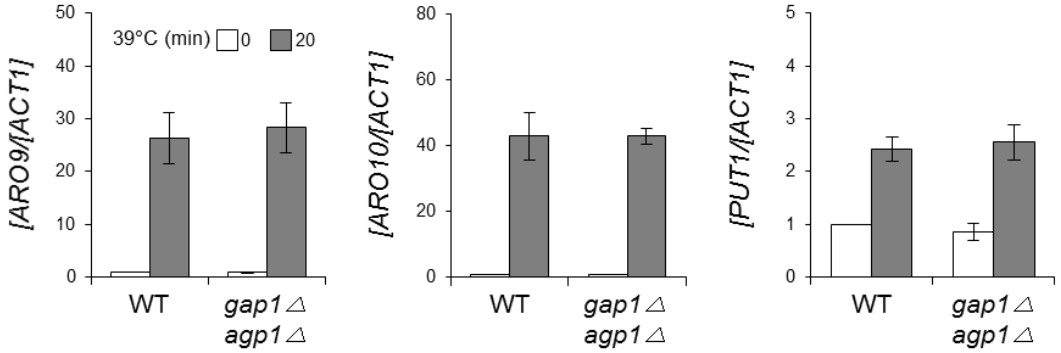

39°C (min) 0 20
[ARO9/[ACT1]
50
40
30
20
10
0
WT
gap1⊿ agp1⊿
[ARO10/[ACT1]
80
60
40
20
0
WT
gap1⊿ agp1⊿
[PUT1/[ACT1]
5
4
3
2
1
0
WT
gap1⊿ agp1⊿

Figure 6.6 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*.

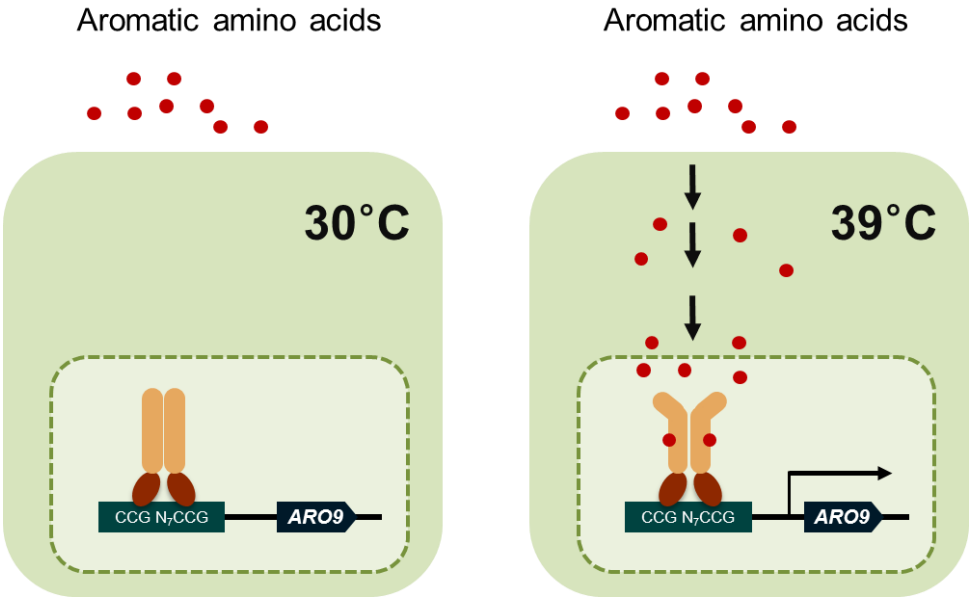


Figure 6.7 Model of heat shock induced expression of *ARO9* and *ARO10*.

Aro80 is constitutively bound the target promoter. Aro80 is inactive at 30°C. In the event of 39°C heat shock condition, influx of aromatic acids in cells leads to the activation of Aro80 and subsequent increase in *ARO9* expression

6.8. Conclusion

In this chapter, it has been reported that Aro80 target genes, *ARO9* and *ARO10*, are induced by heat shock in an Aro80-dependent manner. However, heat shock-related signaling pathways including PKA, PKC, and Hog1 pathways are not involved in the heat shock activation of Aro80. It was elucidated that heat-induced increase in amino acids influx can lead to the inducer-dependent activation of Aro80 upon heat shock. Known aromatic amino acids permeases play a minor role in the heat-induced expression of *ARO9* and *ARO10*, suggesting that increase in plasma membrane fluidity might be responsible for amino acids influx during heat shock.

Chapter 7.

Promoter engineering by using Aro80 binding sites

7.1. Introduction

Metabolic pathway is a sequence of biochemical enzyme reactions. Synthetic regulation of expression of these enzyme genes is important for metabolic engineering parts [30,58,92,93]. One of the key access points to change in metabolic flux is to control mRNA quantity at the promoter level [59]. Therefore, selection of appropriate promoters is required especially for production yield and efficiency optimization [27,52]. Promoter can be defined as specific sequences. It can facilitate the binding of transcription factors and enable transcription initiation. The interactions between promoter sequence and transcription factors facilitate the recruitment of the transcriptional machinery necessary for transcription of a specific gene [49].

Eukaryotic promoters consist of core element and upstream activation sequence (UAS) [49]. The direction and start site of transcription are regulated by the core promoter element, and the upstream activation sequence controls promoter strengths and frequency. Therefore efficient transcription needs coupling of compatible upstream activation sequence and core promoters. Many native promoters have been well-characterized in *S. cerevisiae* [93,94,95,96,97,98,99,100]. Especially, strong endogenous constitutive promoters (including $P_{ADH,}$ P_{TEF}, P_{HXT}, and P_{GPD}) or galactose-inducible promoters P_{GAL} are usually employed for metabolic engineering application [27]. Constitutive promoters offer continuous gene expression levels without the requirement of specific inducers. On the other hand, inducible promoters offer a complementary method for the control of gene expression usually via the addition of inducers. The most widely used inducible promoters for metabolic pathway engineering are the GAL promoters [101]. Previously, it was investigated that P_{GAL}

inducible promoters show tight control with 1,000-fold induction between glucose-repressed and galactose-induced expression [33]. However, P_{GAL} inducible promoters have several problems for industrial application. Firstly, the presence of glucose in growth media represses the expression of Gal promoter. The transcription of P_{GAL} inducible promoters is rapidly induced after culture in non-fermentable carbon sources, where this condition usually supports slow growth rate. Secondly, the intracellular galactose quantity is gradually decreased by cell growth because of consumption as a carbon source. It may lead to reduced expression of P_{GAL} inducible promoters. Thirdly, galactose is an expensive carbon source. It is needed in higher concentration for efficient expression. Therefore cost of galactose influences the final process cost to a large extent. Therefore, galactose promoter is not suitable for a large scale application. Although Gal promoter has higher expression levels of target genes by inducer, strong overexpression is not always optimal for efficient metabolic flux. Therefore a wide range of promoter strengths is required for biotechnological application. Therefore, promoter engineering approach is becoming an emerging tool to facilitate this optimization and offer more synthetic promoter elements. Prior attempts to modulate gene expression include altering endogenous promoter strength through random mutagenesis, saturation mutagenesis of nucleotide spacer regions, direct modification of TFBS (transcription factor binding site) and hybrid promoter engineering. Among these attempts, hybrid promoter engineering has been well-investigated, most recently.

Synthetic hybrid promoters consist of a core promoter region fused to a single upstream activating sequence or tandem upstream activation sequence repeats that modulate promoter strength [33]. These upstream activation sequences help localize specific transcription factors for increase in the transcriptional activity of the core

promoter. Therefore the upstream activation sequence (UAS) and core promoter regions serve as modular synthetic parts that can be combined to produce a strong, functional UAS-core promoter module [33,102].

The expression of *ARO9* and *ARO10* genes are increased by aromatic amino acids via transcriptional activator Aro80. Aromatic amino acids (tryptophan, phenylalanine and tyrosine) convert to aromatic alcohols (tryptophol, phenylethanol and tyrosol) through aminotransferase Aro9 and decarboxylase Aro10 [5]. Among aromatic alcohols, tryptophol, as a quorum sensing molecule, could induce the expression of *ARO9* and *ARO10* in an Aro80-dependent manner. It means that the promoters of *ARO9* and *ARO10* are regulated by positive feedback through substrate (aromatic amino acids) and product (tryptophol) [4]. Previously, the character of a UAS element that is required for induction of the *ARO9* gene has been investigated by using lacZ reporter system [2]. This research demonstrated that the 36 bp sequence contains a UAS element necessary and sufficient for induced expression of lacZ reporter gene in response to tryptophan.

Therefore in this chapter, the synthetic promoter engineering approach was investigated to the yeast *S. cerevisiae* by creating several synthetic *ARO9* promoter variants. Firstly, inducible P_{ARO9}-EGFP plasmid was constructed by isolating 36 bp Aro80 binding site containing *ARO9* core promoter region. Using this construct, tryptophan-dependent expression was observed by EGFP signal. Secondly, *ARO9* promoter variants were constructed by using tandem *ARO9* UAS and high copy number plasmid. These synthetic promoters showed a dynamic range of inducible promoter strengths. Finally, *ARO9* promoter was applied for the production of acetoin and 2, 3-butanediol. To summarize, this study creates tryptophan-inducible promoter

and establishes *ARO9* variant promoters as a synthetic approach to control of promoter strength in *S. cerevisiae*.

7.2. Construction of pARO9-EGFP reporter system

The *ARO9* promoter is induced by tryptophan treatment via Aro80. Also, the 36-bp sequence contains a UAS element necessary and sufficient for induced expression of the fusion gene in response to tryptophan. *ARO9* UAS contains typical consensus binding sequences of Aro80. In this study, by using *ARO9* upstream activation sequence as an activation module, synthetic hybrid promoter was developed. First of all, pARO9-*EGFP* reporter system was constructed to monitor the expression strengths (Fig 7.1 A). And then two and four binding sites of Aro80 were added to the pARO9 original promoter, creating promoters that contain six and eight Aro80 binding sites. Next, a series of *ARO9* core promoter-based synthetic promoters were created by fusing between one and two binding sites of Aro80 to the original *ARO80* UAS. The sequence difference of these promoters is described in figure (Fig 7.1 B).

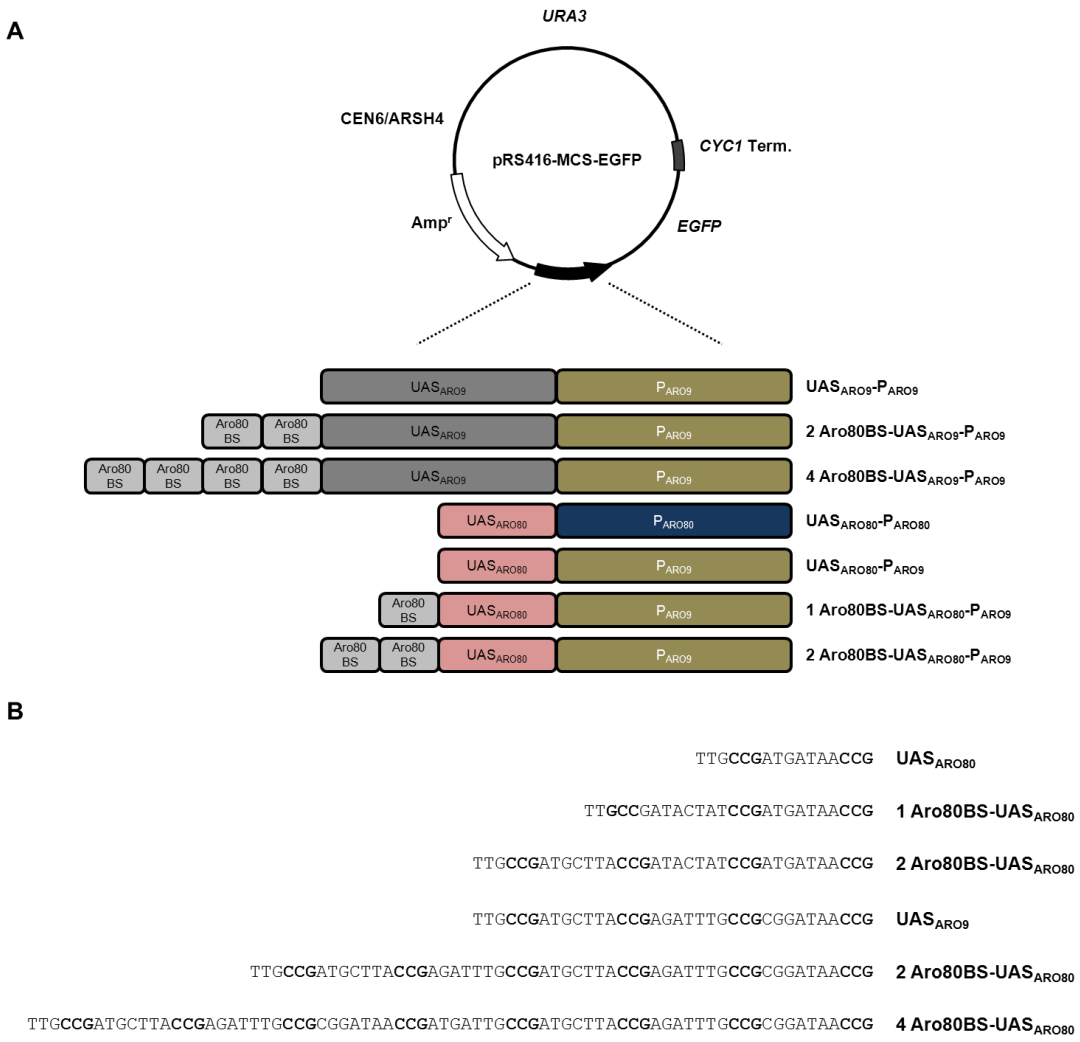


Figure 7.1 Construction of hybrid promoter.

A. A simplified schematic is provided detailing the construction of hybrid promoter-based fluorescence cassettes within the p416-MCS-EGFP plasmid backbone

B. Detailed promoter sequences for the construction of these plasmids

7.3. Investigating the effect of synthetic *ARO9* promoters

Next, the strengths of constructed promoters were observed via EGFP signals. Yeast cells transformed with pARO9-EGFP and pARO9 variant-EGFP were cultured in the presence or absence of tryptophan. Then, EGFP fluorescence signals were observed by using a microplate reader.

The EGFP signal of pARO9-EGFP was increased by treatment of tryptophan in a concentration-dependent manner (Fig 7.2). Therefore the strengths of pARO9-EGFP can be regulated by tryptophan concentration (Fig 7.2).

Next, to determine whether this EGFP signal was caused by the increase in the transcription, the mRNA expression levels were observed by time points after tryptophan treatment. mRNA expression of pARO9-EGFP was gradually increased by culture time without tryptophan (Fig 7.3). On the contrary, tryptophan treatment rapidly induced expression of EGFP at mRNA level. Therefore, increased EGFP signal by tryptophan reflects increase in the transcription from the pARO9 promoter. One property of ARO9 promoter is the induction in relatively short period of time; 1 hr, after the treatment of tryptophan.

Next, the EGFP signals of pARO9 WT, +2 Aro80 BS (binding site) and +4 Aro80 BS were observed. It was expected that promoter strengths of these promoters increase by increasing Aro80 binding sites in the *ARO9* promoter. However, the addition of Aro80 binding sites to original *ARO9* promoter did not enhance the fluorescence levels (Fig

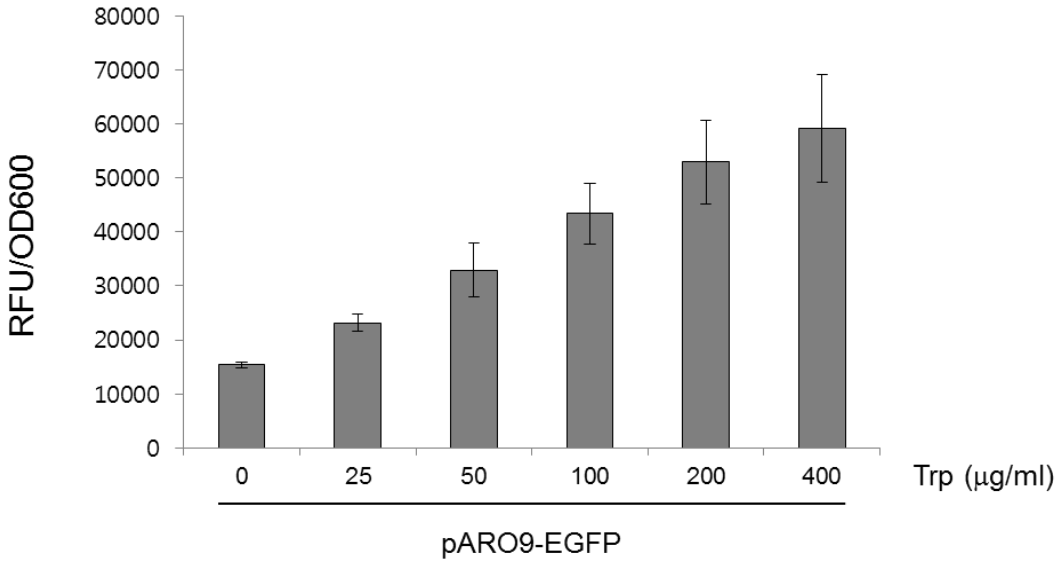


Figure 7.2 Effect of tryptophan concentration on pARO9-EGFP expression.

pARO9-EGFP construct was tested in medium containing various concentrations of tryptophan as inducer.

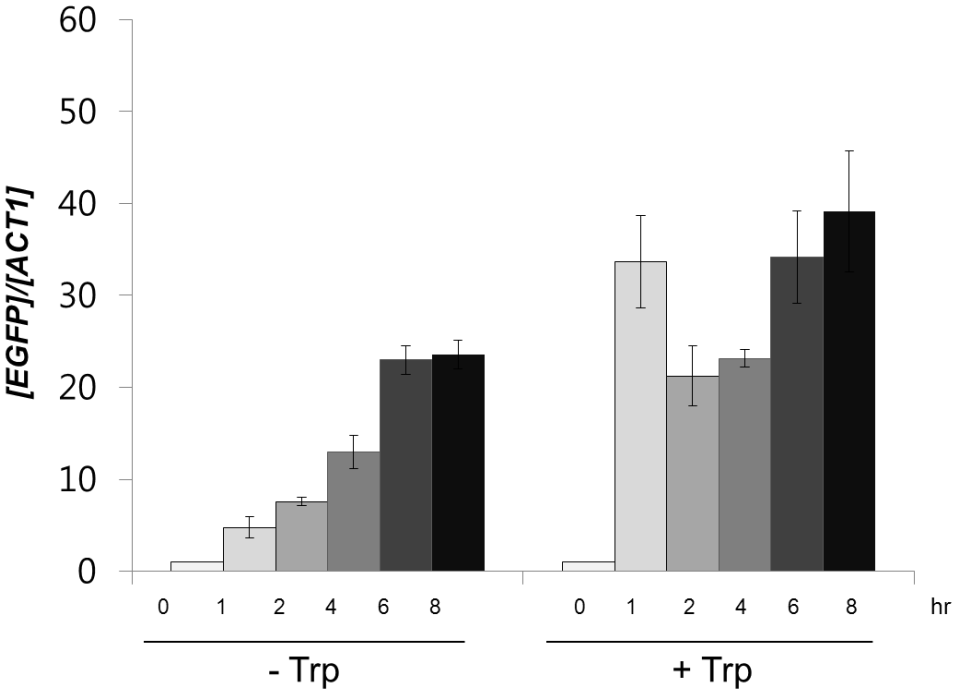


Figure 7.3 mRNA level measurements of pARO9-EGFP construct.

Cells were grown in minimal medium lacking amino acids (− AA) or containing 200 μg /ml tryptophan (+ Trp) until early exponential phase. EGFP mRNA levels were analysed by qRT-PCR normalized with *ACT1*. Each value represents the average ± standard deviation from three independent experiments.

7.4). Interestingly, EGFP signals were decreased by the addition of Aro80 binding sites to the original *ARO9* promoter. One possibility of this result is that increased binding sites of *ARO9* promoter might act as a competition sites of Aro80, preventing its functional binding for transactivation. Therefore EGFP signals of pARO9 WT, +2 Aro80 BS and +4 Aro80 BS were detected in Aro80-overexpressed yeast cells. In this case, Aro80 binding site-dependent reduction in EGFP signal was abolished, supporting hypothesis of competition binding (Fig 7.5). Although the increase in the strength of *ARO9* variant promoter could not been developed by the addition of Aro80 binding sites to *ARO9* original promoter, reduction in the strength of promoters were found by this experiment. These results demonstrate a possible usage of the variant promoters in the reduction of expression. Although the promoter sequences of *ARO9* and *ARO80* are very similar, the expression of *ARO80* was not affected by tryptophan. The difference of *ARO80* and *ARO9* promoters is the number of Aro80 binding site; CCG triplet. The promoter of *ARO80* has two Aro80 BS. On the contrary, *ARO9* promoter contains four Aro80 BS. Therefore, it was investigated whether the promoter strength of *ARO80* promoter was affected by the addition of Aro80 binding site to the original *ARO80* UAS. However EGFP signals of UAS_{ARO80} WT, +1 Aro80 BS and +2 Aro80 BS were not increased by tryptophan treatment. This result means that *ARO80* promoter does not work properly as a tryptophan inducible promoter.

As a next step, synthetic hybrid promoter engineering was performed by using Aro80 binding site and the *ARO9* core promoter. One or two Aro80 binding sites of original *ARO80* promoters were fused to the *ARO9* core promoter. Then the EGFP signals of UAS_{ARO80} WT, +1 Aro80 BS and +2 Aro80 BS were observed. The promoter strengths of *ARO80* hybrid promoters were increased by increasing Aro80 binding sites (Fig 7.6).

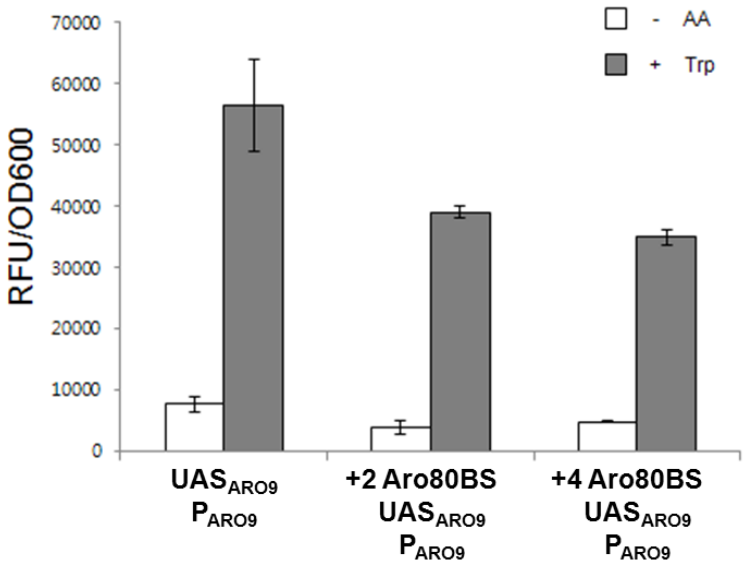


Figure 7.4 Promoter strengths of *ARO9* promoter variants.

Relative fluorescence of EGFP *ARO9* promoter variants. Cells were grown in minimal medium lacking amino acids (− AA) or containing 200 μg /ml tryptophan (+ Trp) until early exponential phase. Error bars represent standard deviation from biological triplicates.

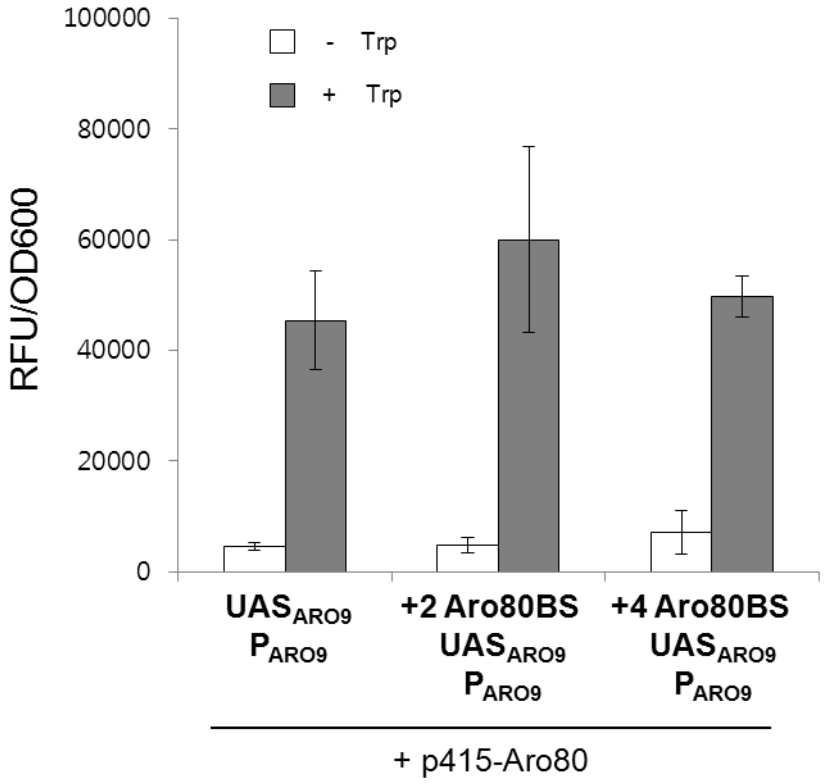


Figure 7.5 Promoter strengths of *ARO9* promoter variants in Aro80 over-expressed cells.

Relative fluorescence of EGFP *ARO9* promoter variants under tryptophan treatment. Cells were grown in minimal medium lacking amino acids (− AA) or containing 200 μg /ml tryptophan (+ Trp) until early exponential phase. Error bars represent standard deviation from biological triplicates.

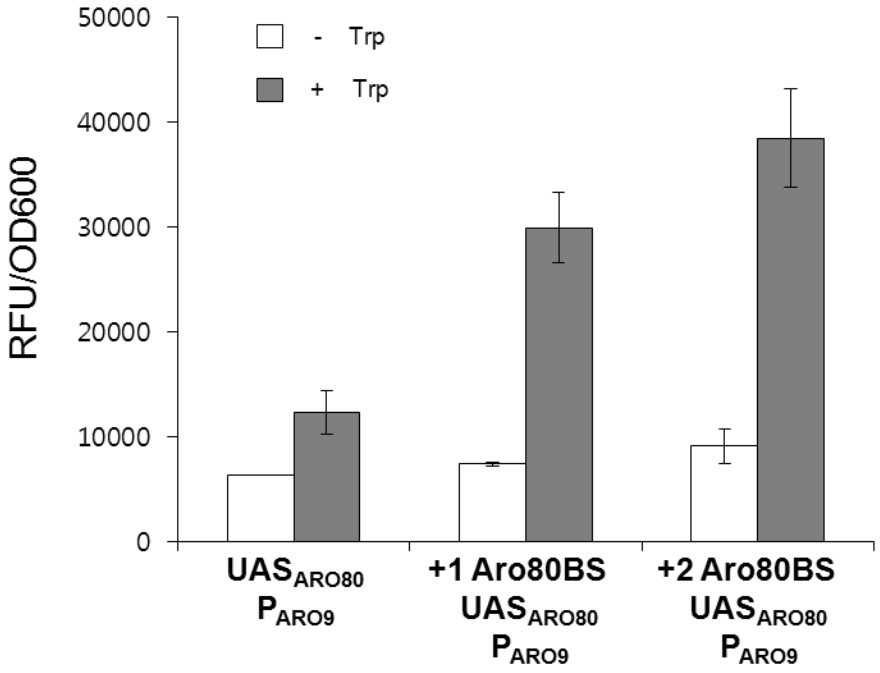


Figure 7.6 Promoter strengths of pARO80 and cARO9 hybrid promoters.

Relative fluorescence of EGFP pARO80-cARO9 hybrid promoter variants under tryptophan treatment. Cells were grown in minimal medium lacking amino acids (− AA) or containing 200 μg /ml tryptophan (+ Trp) until early exponential phase. Error bars represent standard deviation from biological triplicates.

Although, addition of $UAS_{ARO80,}$ containing two Aro80 binding sites, to the *ARO9* promoter showed no increase in induction, addition of one or two Aro80 binding sites allowed tryptophan-dependent induction, suggesting that at least three Aro80 binding sites are required for induction. In addition, these results suggest that employing different number of Aro80 binding sites as hybrid enhancer elements enables differential levels of expression. CEN6 (low copy number) and 2 micron-based (high copy number) expression plasmids are widely used in yeast for the expression of recombinant protein. It has been demonstrated that the regulation of plasmid copy number is important for the optimization of metabolic pathway. Therefore the EGFP signals of *ARO9-EGFP* promoter were detected by using low and high copy plasmid. From the result, higher EGFP signals were observed in high copy-based pARO9-EGFP promoter (Fig 7.7). However, one minor drawback of this system is that it also showed increase in the basal expression without tryptophan. In promoter engineering, a tight regulation in inducer-specific induction is required, thus further study is needed to solve this problem.

Taken together, the concentration of tryptophan, the number of Aro80 binding site and plasmid copy number can regulate the strengths of *ARO9* promoter. Moreover, combining these factors would provide a wide range of gene expression levels useful for synthetic biology and metabolic engineering.

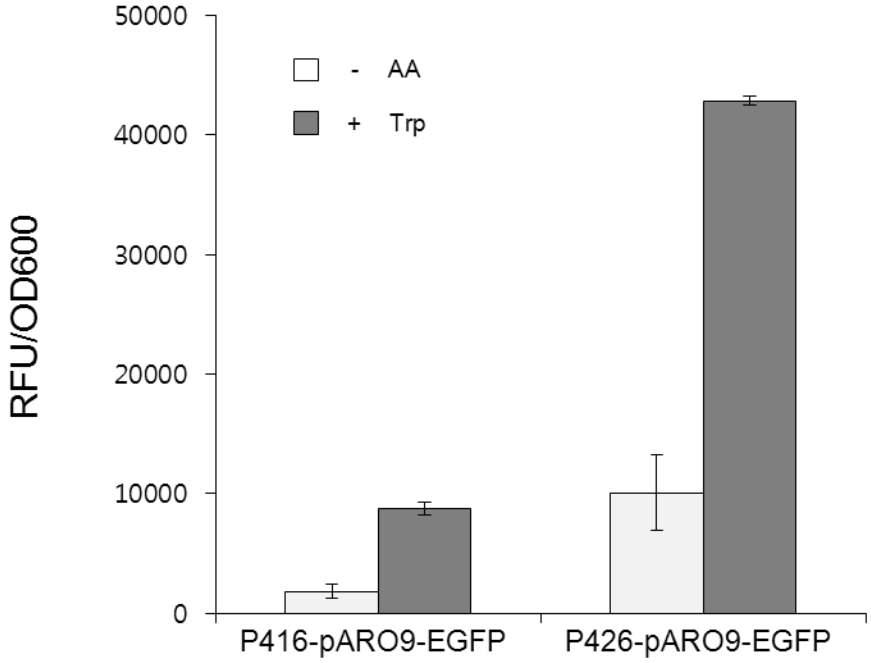


Figure 7.7 Effect of plasmid copy number on the strength of *ARO9* promoter.

Relative fluorescence of low and high copy pARO9-EGFP under tryptophan treatment. Cells were grown in minimal medium lacking amino acids (− AA) or containing 200 μg /ml tryptophan (+ Trp) until early exponential phase. Error bars represent standard deviation from biological triplicates.

7.4. Application of the tryptophan inducible *ARO9* promoter for acetoin and 2, 3-butanediol production

In *B. subtilis*, pyruvate is converted into acetoin via α-acetolactate by acetolactate synthase (AlsS), α-acetolactate decarboxylase (AlsD). Subsequently, acetoin is reduced to 2, 3- butanediol by butanediol dehydrogenase (Bdh). It was recently reported that the 2, 3-butanediol biosynthetic pathway from *B. subtilis* was introduced into the yeast strain to produce 2, 3- butanediol from glucose in *S. cerevisiae* [103].

Results in this chapter showed that the strength of pARO9-EGFP plasmid can be further modified by combining the concentration of tryptophan, the number of Aro80 binding sites and the copy number of plasmid. To demonstrate the practical application of this pARO9 promoter, the plasmid carrying the pARO9-alsS and pARO9-alsD was tested for production of acetoin and 2, 3 butanediol in *S. cerevisiae*. Because EGFP signals of p*ARO9* were affected by tryptophan concentration, pARO9-AlsS and pARO9-AlsD transformed yeast cells are cultured with 50 μg/ml and 500 μg/ml tryptophan and without tryptophan (Fig 7.8). Then, the productions of acetoin and 2, 3-butanediole were detected by gas-chromatography. The productions of acetoin and 2, 3-butanediol were increased with the increase in concentration of tryptophan, suggesting that AlsS and AlsD transcription levels were successfully controlled by tryptophan induction.

In yeasts, there are a limited number of available inducible promoters that can be applied to metabolic engineering. Hence, this study shows a possibility of *ARO9* promoter as a tryptophan inducible promoter that is applicable to metabolic engineering.

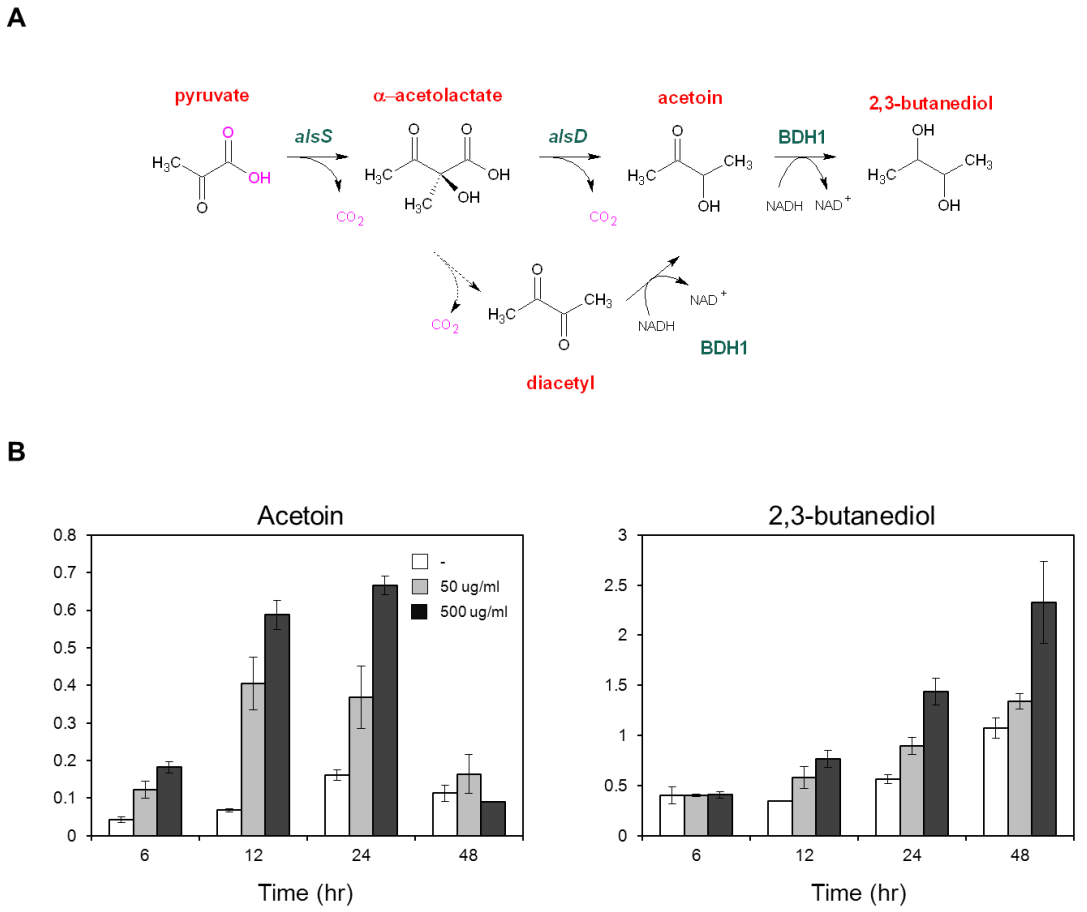


Figure 7.8 Effect of tryptophan concentration on product formation by pARO9 promoter.

Acetoin and 2, 3-BD production by pARO9-ALSS and pRO9-ALSD were examined under aerobic conditions by increasing tryptophan concentration.

This newly designed tryptophan-inducible *ARO9* promoter is useful for metabolic engineering that aim at optimal production of various valuable products using *S. cerevisiae*.

7.5. Conclusion

In this chapter, tryptophan-inducible synthetic *ARO9* promoters were developed. It was shown that the concentration of inducer (tryptophan) and plasmid copy number can control the strength of *ARO9* promoter via regulation of transcription level. This study further enabled fine-tuned tryptophan-induced expression by fusing Aro80 binding sites to *ARO9* core promoter. Especially, Aro80 binding sites can act as modular, synthetic transcriptional amplifiers. Production of acetoin and 2, 3 butanediol by using *ARO9* promoter shows the possibility of biotechnological application. This study enabled the production of inducible promoters in *S. cerevisiae* and has created a range of expression capacities.

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Chapter 8.

Discussion

Most of the genes involved in utilization of alternative nitrogen sources are regulated by both pathway-specific regulators and GATA factors governing global regulation. In this thesis, the interplay of Aro80, a pathway-specific regulator, and GATA factors was elucidated in expression of the *ARO9*, *ARO10* and *ARO80* genes which are involved in catabolism of aromatic amino acids.

Aro80 is constitutively bound to its target promoters and activated by aromatic amino acids

Aromatic amino acids are known as inducers for Aro80. It was shown that Aro80 is constitutively localized in the nucleus and bound to the promoters even in the absence of inducers, suggesting that Aro80 might be activated at the level of transcriptional activation. Such an activation mechanism is also conserved in Put3, a well-studied transcriptional activator of proline utilization pathway. It has been shown that proline can directly bind to Put3, inducing its conformational changes, which might unmask the C-terminal activation domain of Put3 [70]. Although the direct binding of aromatic amino acids to Aro80 has not yet been demonstrated, it is highly likely that aromatic amino acids might also act as ligands to activate Aro80 which is already bound to DNA. However, such a constitutive binding is not conserved in all Zn_2Cys_6 family of transcription factors involved in catabolism of various nitrogen sources. For example, Uga3, a pathway-specific transcription factor for GABA catabolism, binds to its target promoters in a GABA-dependent manner along with Dal81, a pleiotropic activator [13,104]. It was reported that branched chained amino acids and methionine also induce the expression of *ARO9* and *ARO10* [9]. Therefore it will be further

investigated whether Aro80 is required for the transcriptional activation of *ARO9* and *ARO10* by branched chained amino acids and methionine. It was known that transaminase Aro9 and decarboxylase Aro10 enzymes have broad substrate specificity and are induced by various amino acids such as aromatic amino acids, branched amino acids and methionine. It is possible that their broad substrate specificity may contribute to reduce the cellular energy for nitrogen accumulation under nitrogen starvation condition.

It was reported that *ARO9* and *ARO10* are important for the production of aromatic alcohols. It should be noted that *ARO9* and *ARO10* genes are involved in quorum sensing phenomenon via positive feedback production of tryptophol as a quorum sensing molecule [4]. In stationary phase, tryptophol accumulates extracellularly as a quorum sensing molecule and activates transcription of *ARO9* and *ARO10* via transcription factor Aro80. Therefore, the result from this study can contribute to understanding yeast quorum sensing. Recently, a research of 2-phenylehanol production was performed based on the regulation mechanism of *ARO9* and *ARO10* by Aro80 and ligands [105]. And production of 2-phenylehanol was successfully increased about 70 fold. It means that the results of this study can contribute to metabolic engineering field for aromatic alcohol production.

Interplay between Aro80 and GATA factors in regulation of Aro80 target genes.

Previously, it has been shown that Gat1 and Gln3 contribute to derepression of P_{ARO9}-

lacZ by poor nitrogen source (urea) in the presence of tryptophan inducer [2]. However, the role for GATA factors was predicted to be indirectly involved in the regulation of inducer uptake. Unlike the previous prediction, our ChIP experiments have revealed direct binding of Gat1 and Gln3 to the *ARO9*, *ARO10*, and *ARO80* promoters upon rapamycin treatment. Interestingly, the results of this study have shown that Aro80 is absolutely required for recruitment of Gat1 to the Aro80 target promoters. Gln3 also showed Aro80-dependency in DNA binding although to a lesser extent. Transactivational activity of Aro80 seems to be not necessary for inducing GATA factor binding to the promoters. In previous studies, the -168 to -133 region of *ARO9* promoter containing four CCG sequences and one GATAA sequence was shown to be sufficient for GATA factor-dependent nitrogen regulation [2]. Therefore, Aro80 and Gat1 or Gln3 might bind to this region with close proximity especially if all four CCG half sites are occupied by two molecules of Aro80 dimer. Although protein-protein interaction between Aro80 and Gat1 was not confirmed in this study, it is possible that rapamycin-dependent physical interaction between Aro80 and GATA factors could affect the recruitment of GATA factors to Aro80 target genes. Unlike Aro80, Put3 is not absolutely required for rapamycin-dependent induction of *PUT1*. However, phosphorylation of Put3 in the presence of rapamycin or poor nitrogen sources has shown to facilitate association of Gat1 to the *PUT1* promoter. Since rapamycin-dependent induction of Aro80 target genes is not completely abolished in *gln3Δgat1Δ*, it is possible that Aro80 might also be activated by TORC1 inhibition. Aro80 activation upon rapamycin treatment could be an indirect effect caused by increased intracellular levels of aromatic amino acids. However, it is also possible that yet, unidentified rapamycin-dependent modification of Aro80 could affect the

recruitment of GATA factors to the promoters. Although several Zn_2Cys_6 family proteins are known as phosphoproteins, it still needs further studies to clarify whether Aro80 is also regulated by phosphorylation in response to different nitrogen sources. Nitrogen regulation of most known NCR-sensitive genes occurs even in the absence of inducers. On the contrary, induction of P_{ARO9}-lacZ expression in urea medium was observed only in the presence of tryptophan [2]. Likewise, it was reported that P_{ARO9}-lacZ was not induced by rapamycin in synthetic medium lacking amino acids. However, based on results showing Gat1 binding to the Aro80 target promoters irrespective of inducer availability, the apparent lack of nitrogen regulation in the absence of inducers might be caused by relatively weak contribution of GATA factors in activation of Aro80 target genes. *ARO9* expression levels increased gradually as increasing Trp concentration in minimal medium, while rapamycin treatment induced about 2- to 3- fold increase in *ARO9* expression, irrespective of tryptophan concentration. Therefore, inducer availability might be a key determinant for expression of Aro80 target genes, and Gat1 and Gln3 can further activate transcription in the absence of preferred nitrogen sources or upon TORC1 inhibition. This regulatory mechanism might allow expression of Aro80 target genes primarily depending on substrate availability for the catabolic pathway.

Beside the regulation of positive GATA factors, expression of NCR gene was regulated by negative GATA factors Gzf3 and Dal80 [1,17]. Gzf3 and Dal80 bind to GATAA sequence of promoters. Because zinc finger domain of Gzf3 and Dal80 are highly homologous to positive GATA factors, it was assumed that negative GATA factors repress by competing with Gln3 or Gat1 for the GATAA sites located in the promoters of NCR genes. It was reported that negative GATA factors play different

roles in the regulation of NCR gene expression [1]. Gzf3 inhibits expression levels of NCR genes under nitrogen repression. In contrast, Dal80 limits the activation of NCR genes under non-preferred nitrogen source. Previous studies show that the deletion of either *GZF3* or *DAL80* modestly increased *DAL5* expression compared to the wild type under rapamycin treatment or poor nitrogen source [81]. The results of this study show that Dal80 and Gzf3 are not involved in expression of *ARO9* and *ARO10* under rapamycin treatment or poor nitrogen source. Although many of NCR genes are regulated both positive GATA factors and negative GATA factors, negative GATA factors do not affect the expression of *ARO9* and *ARO10.*

It has been known that Sit4 and PP2A phosphatases act as downstream effectors of TORC1 in regulation of GATA factors [41]. The results of this study have shown that PP2A, but not Sit4, is involved in rapamycin-dependent induction of Aro80 target genes, possibly by regulating Gat1 and Gln3. These results are consistent with the recently identified intranuclear function of PP2A in rapamycin-induced GATA factor binding to the *DAL5* and *GDH2* promoters [80]. Although the detailed mechanisms for the PP2A-dependent regulation of GATA factors remain to be elucidated, these results suggest that the prevailing Sit4-dependent regulation of Gln3 localization might be only a part of the more complicated mode of TORC1-dependent regulation of GATA factors.

In summary, the results of this study have shown that transcription of genes involved in catabolism of aromatic amino acids is regulated by mutual regulation between Aro80 and GATA activators. Aro80 is required for GATA factor binding to the Aro80 target promoters, while GATA factors can indirectly influence Aro80 activity through

regulating import of Aro80 inducers. Therefore, GATA factors might play both direct and indirect roles in transcriptional regulation of Aro80 target genes.

Heat shock activates transcription factor Aro80 by heat-induced aromatic amino acid influx in *Saccharomyces cerevisiae*

In this thesis, it was suggested that 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. It was known that detergents are important factors for membrane fluidity. Therefore several detergents might stimulate uptake of amino acids by regulating membrane fluidity. 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.

Promoter engineering by using Aro80 binding sites

A dynamic range of well-controlled and tunable promoters are required for metabolic engineering in *S. cerevisiae*. Here, promoter engineering approach for the creation of a

novel inducible promoter in *S. cerevisiae* was applied. The utility of this approach with three main studies were demonstrated. First, the dynamic range of tryptophan inducible promoters was established by using *ARO9* promoter. Second, the capacity to impart synthetic regulation through a hybrid promoter approach by adding Aro80 binding site to UAS_{ARO80}-P_{ARO9} promoter was demonstrated. Results of this study show that strengths of engineered tryptophan inducible promoter are regulated by tryptophan concentration and the number of Aro80 binding site. Third, tryptophan inducible *ARO9* promoter was applied for the production of acetoin and 2, 3-butanediol.

The newly developed *ARO9* synthetic promoter would be a useful tool to engineer yeast cells for metabolic engineering and synthetic biology. Further optimization of *ARO9* promoter is expected for it to be used in biotechnological application.

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Abstract in Korean

국문 초록

출아성 효모(*Saccharomyces cerevisiae*)는 방향족 아미노산을 질소원으로 이용할 수 있으며, 방향족 아미노산(tryptophan, phenylalanine, tyrosine)은 이화과정을 통하여 방향족 알코올 (tryptophol, phenylethanol, tyrosol)로 변환된다. 방향족 아미노산으로부터 방향족 알코올의 생산은 transamination, decarboxylation, reduction의 3단계 과정으로 구성되는 Ehrlich pathway를 통하여 이루어지며, 아미노산은 이 과정을 통하여 순차적으로 α-keto acid, 알데히드, 알코올로 변환 된다. *S. cerevisiae*에서 방향족 아미노산의 Ehrlich pathway 에 관여하는 transaminase와 decarboxylase는 *ARO9*과 *ARO10* 유전자로 알려져 있다. 본 연구에서는 유도체에 의하여 활성이 조절되는 전사조절인자 Aro80과 질소 원의 양과 질에 의하여 활성이 조절되는 전사조절인자 GATA factors에 의한 *ARO9*과 *ARO10* 유전자의 발현 기작을 전사 수준에서 규명하였다.

첫 번째로 유도체에 의존적인 Aro80 활성화 기작에 대하여 연구하였다. 전사조절인자 Aro80는 핵 안에 위치하며, 유도체의 존재 유무는 Aro80의 세포 내 위치에 영향을 주지 않음을 밝혔다. 또한 Aro80은 목적 유전자의 프로모터에 결합한 상태로 존재하며, 유도체의 존재가 프로모터 결합력에 영향을 주지 않음을 규명하였다. 추가적으로 Aro80는 *ARO80* 유전자의 프로모터에 결합을 할 수 있으나, 유도체에 의한 *ARO80* 유전자의 발현에 영향을 주지 않음을 밝혔다.

두 번째로 *ARO9*, *ARO10* 그리고 *ARO80* 유전자 발현이 Gln3와 Gat1을 포함하는 GATA factor에 의하여 직접적으로 조절됨을 규명하였다. Rapamycin 처리를 통하여 TORC1이 비활성화된 조건에서 Gat1이 *ARO9*,

ARO10 그리고 *ARO80* 유전자의 프로모터에 결합이 증가함을 밝혔고, 이는 Aro80 전사조절인자에 의존적으로 이루어짐을 확인 하였다. 동일한 조건에서 Gln3는 부분적으로 Aro80을 필요로 하였다. 또한 유도체에 의한 Aro80의 활성 변화는 Gln3와 Gat1의 Aro80 목적유전자 프로모터로의 결합에 영향을 주지 않았다. Rapamycin에 의존적으로 조절되는 Aro80의 목적 유전자의 발현에는 TOR의 하위 신호 전달 체계인 Pph21/22 탈 인산화 복합체가 필요하며, Sit4 탈 인산화 효소는 영향을 주지 않음을 규명하였다.

세 번째로 열충격 스트레스에 의하여 *ARO9*과 *ARO10* 유전자의 발현이 증가하며, 이 또한 Aro80에 의존적으로 조절됨을 확인하였다. 먼저 열충격에 의한 Aro80의 활성화는 열충격 스트레스와 관련된 신호전달 체계인 PKA, PKC, HOG에 영향을 받지 않음을 확인하였고, 열충격 스트레스에 의해 세포 내로 유입된 방향족 아미노산이 유도체에 의한 Aro80의 활성 증가를 유도함을 밝혔다. 이와 함께 기존에 알려진 방향족 아미노산 퍼미에이즈(permease)는 열충격 스트레스에 의한 *ARO9*, *ARO10* 유전자의 발현에 영향을 주지 않음을 확인하였다. 종합적으로 열충격 과정에서 증가된 세포막의 유동성은 세포 내부로의 방향족 아미노산의 유입을 유도하고, 이를 통해 활성화된 Aro80이 *ARO9*, *ARO10*의 유전자 발현을 증가시킨다는 모델 제시하였다.

네 번째로 *ARO9*의 프로모터를 기반으로 한 새로운 유도성 프로모터를 제시하였다. *ARO9* 또는 *ARO80* 코어 프로모터에 Aro80 결합 위치의 개수를 조절함으로써 다양한 발현 세기를 갖는 프로모터를 제작하였다. 이 프로모터들의 유전자 발현 수준은 트립토판의 농도를 통하여 조절 할 수 있다. 본 연구를 통하여 제시된 트립토판 유도 프로모터는 대사 공학에서 유전자 발현 조절의 용도로 활용 될 수 있을 것이라 기대한다.

종합적으로 본 연구에서는 유도체와 다양한 환경적 요인에 의하여 *ARO9*과 *AR010* 유전자의 전사 활성이 조절되는 기작을 규명하였고, 이를

기반으로 *ARO9* 프로모터를 이용한 유도성 프로모터의 제작 가능성을 제시하였다.

주요어: 방향족 아미노산 이화과정, nitrogen catabolite repression, Aro80, GATA factor, 열충격, 프로모터 엔지니어링

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