

Response of *Saccharomyces cerevisiae* to Ethanol Stress Involves Actions of Protein Asr1p

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During the fermentation process of *Saccharomyces cerevisiae*, yeast cells must rapidly respond to a wide variety of external stresses in order to survive the constantly changing environment, including ethanol stress. The accumulation of ethanol can severely inhibit cell growth activity and productivity. Thus, the response to changing ethanol concentrations is one of the most important stress reactions in *S. cerevisiae* and worthy of thorough investigation. Therefore, this study examined the relationship between ethanol tolerance in *S. cerevisiae* and a unique protein called alcohol sensitive RING/PHD finger 1 protein (Asr1p). A real-time PCR showed that upon exposure to 8% ethanol, the expression of *Asr1* was continuously enhanced, reaching a peak 2 h after stimulation. This result was confirmed by monitoring the fluorescence levels using a strain with a green fluorescent protein tagged to the C-terminal of Asr1p. The fluorescent microscopy also revealed a change in the subcellular localization before and after stimulation. Furthermore, the disruption of the *Asr1* gene resulted in hypersensitivity on the medium containing ethanol, when compared with the wild-type strain. Thus, when taken together, the present results suggest that *Asr1* is involved in the response to ethanol stress in the yeast *S. cerevisiae*.

Keywords: *Saccharomyces cerevisiae*, ethanol tolerance, gene knockout and tagging, translocation

Organisms have evolved multiple strategies to maintain normal cellular functions in the face of variable and stressful external environments. For example, multicellular organisms have special organs and/or tissues to ensure a

relatively stable internal environment, whereas unicellular organisms, such as the yeast *Saccharomyces cerevisiae*, employ an array of stress response pathways to adapt to drastic environmental changes [20, 22, 27]. Indeed, yeast cells are invariably exposed to a series of stresses, such as high concentrations of ethanol, a high osmolarity, nutrition limitations, freezing, oxidation, and desiccation. For instance, concentrations of ethanol above 4%–6% can severely affect the normal processes within yeast cells, and previous investigations have revealed several strategies used by yeast cells to survive in the presence of high ethanol concentrations, including initiating a common gene expression program, changing the membrane composition, and increasing the chaperone proteins [1].

Several nuclear transcriptional factors involved in the stressful pathway have also been suggested to be related to the cellular responses to elevated ethanol in *S. cerevisiae*. Heat shock factors (HSFs) are a group of highly conserved regulating factors of heat shock proteins (HSPs), and are in charge of the response to elevated temperatures and a variety of chemical and physiological stresses. It has already been shown that treatment with 6% ethanol induces the formation of the active conformation of Hsf1 (the only HSF in *S. cerevisiae*) [17]. In addition, to activate HSPs under stressful conditions, HSFs can also regulate the expression of other stress response genes specific to ethanol stimulation. For example, an endoplasmic reticulum oxidoreductin encoded by the *ERO1* gene involved in the formation of protein disulfide bonds has also been revealed to be up-regulated by Hsf1 [26]. The STRE (stress responsive element) pathway, which is mediated by the transcription factor Msn2p and its homologous Msn4p (also called Msn2/4p), can also activate gene expression in the case of exposure to a variety of other generally adverse stimuli, such as a low pH, heat shock, nutrient limitations, oxidative stress, and an increased ethanol concentration. The target genes regulated by Msn2/4p include proteins

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Table 1. Yeast strains used in this study.

Strain	Genotype	Source
S288C	<i>MAT</i> alpha SUC2 mal mel gal2 CUP1 flo1 flo8-1 hap1	ATCC collection
S288C- <i>Δasr1</i>	<i>MAT</i> alpha SUC2 mal mel gal2 CUP1 flo1 flo8-1 hap1 <i>Δasr1</i>	This study
S288C- <i>GFP</i>	<i>MAT</i> alpha SUC2 mal mel gal2 CUP1 flo1 flo8-1 hap1 <i>GFP</i>	This study

involved in stress protection functions, such as *DDR2*, *CTT1*, *HSP12*, *TPS1*, and *TPS2*, some of which have also been revealed to participate in the ethanol response [18, 24, 25]. Furthermore, the signaling pathways, such as the HOG pathway, cyclic AMP (cAMP)-dependent protein kinase A (PKA) pathway, Tor (target of rapamycin) signaling pathway, HSE-mediated transduction pathway, and stress-activated MAP kinase pathway, have all been proposed to participate in the cell reactions to different stressful conditions mediated by Msn2/4p [12, 13, 21]. However, whether the signaling pathways described above play a role in the ethanol response remains unclear, or there may be some other potentially unknown pathways related to ethanol stress in *S. cerevisiae*.

Accordingly, in an attempt to discover and characterize the function of ethanol stress response genes in *S. cerevisiae*, this study focused on a 36 kDa (*YPR093C*) stress responsive protein Asr1p (alcohol sensitive RING/PHD finger 1 protein) that was previously suggested to be involved in the ethanol stressful response [5]. Upon exposure to ethanol, this protein changes its subcellular localization and translocates from the cytoplasm to the nucleus. However, unexpectedly, Asr1p functions as a classical transcription factor, like Msn2/4p or Hsf1, when it enters the cell nucleus. Asr1p harbors two N-terminal RING/PHD fingers, yet contains no DNA-binding domains. Thus, in contrast to the well-known description of its mechanism related to nucleocytoplasmic trafficking, there is still limited knowledge of the function of Asr1p in relation to ethanol-induced stress. Furthermore, no definitive

evidence of the role of Asr1p in the ethanol stress response has yet been established, as no phenotypic differences have been found between wild-type and Asr1-deficient strains during *sake* brewing or wine-making [15].

However, this study found conclusive evidence of a relationship between Asr1p and ethanol stress, and discovered its potential function in the ethanol response in *S. cerevisiae*. Real-time PCR showed that the expression level of *Asr1* changed upon ethanol exposure. A localization experiment with fluorescence-tagged Asr1p showed that it shuttled between the cytoplasm and the nucleus. Yeast cells with an *Asr1* deficiency exhibited retarded growth when compared with the wild-type cells with an ethanol concentration above 8%. Thus, the experimental results from this study substantially indicated that Asr1p is involved in the ethanol stress response in yeast.

MATERIALS AND METHODS

Yeast Strains, Medium, and Plasmids

The yeast strains used in this study are listed in Table 1. The *S. cerevisiae* strain S288C used in this study was purchased from ATCC (American Type Culture Collection), and its whole genome sequence has already been completed and published [11]. To construct the *Asr1* gene knockout and Asr1-GFP fusion strains, the PCR products used for the homologous recombination were generated using either pFA6a-kanMX4 or pFA-GFP (S65T)-kanMX6 as the template [28, 29]. The yeast cells were generally grown on rich YPD medium (1% yeast extracts, 2% peptone, 2% glucose), plus 350 mg/l geneticin sulfate (G418) (Ameresco) when positive

Table 2. Primers used in this study.

Primer name	Primer sequence (5' → 3')
P1	GTGAACAATTTGGATGTTTGAACGTTTGCGGGCATAAATT- <u>AGCTTCGTACGCTGCAG</u>
P2	GAGTATTATACTGTTTATATCTTGTTTTAATGTTATATTGCATTAAT- <u>CGATGAATTCGAGCTCG</u>
P3	GAAGTGGAGTGCACACAGAAGTCTTAATTTACTGTCATGATGAG- <u>CGTACGCTGCAGGTTCGAC</u>
P4	CATACAAGGCTGAACTTAACGAAAAAGGGTTGTTCGACAT- <u>ATCGATGAATTCGAGCTCG</u>
P5	TGCGTCATTGACTCCAACAT
P6	ACCGAGGCAGTTCCATAGG
P7	TGTCTACGCGGGCTAGCTT
P8	AGAACCTCAGTGGCAAATCCT
Asr1(F)	TGTATCGTCTCATTTCTTTTCGTT
Asr1(R)	ATTTAGAAGTTGGCGAAGGGC
Actin(F)	CCCAGGTATTGCCGAAAGAATGC
Actin(R)	TGGAAGGTAGTCAAAGAAGCCAAG

Underlined sections of primers denote amplified fragments from plasmids pFA6a-kanMX4 or pFA6a-GFP (S65-T)-KanMX6.

transformants were screened. The strains were kept in 20% glycerol at -80°C or on YPD plates at 4°C .

Real-Time PCR

An overnight culture of *S. cerevisiae* S288C was grown in YPD and 1% (v/v) of this culture was used to inoculate six flasks containing 50 ml of YPD. Once the yeast cells reached an $\text{OD}_{600}=0.5\text{--}0.8$ ($\sim 1.5 \times 10^7$ cell/ml) at 30°C , 8% ethanol was added to the samples. The cells were then harvested by centrifugation at 0 min, 30 min, 60 min, 120 min, 240 min, and 360 min after ethanol exposure. Total RNA was isolated using an RNA mini-extraction kit (Tiangen Co.) and the genomic DNA then eliminated using a TUBE DNA-free kit (Biotek). The amount of total RNA was quantified by monitoring the absorbance at 260 nm. The synthesis of cDNA was carried out using a Reverse Transcriptase kit (Takara) according to the manufacturer's instructions. The real-time PCR analysis was performed using a SYBR Green PCR kit (Sigma Co), and the primers used are shown in Table 2: Asr1(F) and Asr1(R) were used to amplify the targeted fragment of the *Asr1* gene, whereas Actin(F) and Actin(R) were used to amplify the actin gene as the control.

The real-time PCR was performed in MicroAmp optical 96-well reaction plates (Applied Biosystems, Inc.) using an ABI Prism 7700 sequence detection system with 1 cycle at 95°C for 10 min and 40 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. All the samples were tested in triplicate. The data were analyzed using the Sequence Detection System Software (SDS 2.2.2; ABI) [8, 30]. The differences in the gene expression were calculated using the comparative CT method.

Asr1 Gene Disruption and Asr1p-GFP Tagging

The deletion of the *Asr1* gene and construction of the C-terminal green fluorescent protein (GFP)-tagged Asr1p were carried out by a PCR-based method using disruption cassettes derived from the plasmids pFA6a-kanMX4 and pFA6a-GFP (S65T)-KanMX6 [3, 7, 29]. The dominant resistance marker kanMX4 was amplified by PCR using primers containing homologous sequences to the target ORF's flanking sequences. The primers used in this study are listed in Table 2: among them, P1 and P2 were used to amplify the gene knockout fragment; P3 and P4 were used to amplify the Asr1-GFP tagging combination; P5 and P6 were the verification primers for the mutants of the *Asr1* gene deletion; and P7 and P8 were used to select the Asr1-GFP fusion mutants. EX *Taq* polymerase (Takara Co., Japan) was employed to amplify the fragments for the homologous recombination. The DNA cloning techniques were performed using standard procedures. The transformation protocol was based on the LiOAc method [16, 23]. The antibiotic G418 was used to select the positive transformants.

After the initial screening, Southern hybridization was also used to confirm the successful construction of the mutants. The genomic DNA from yeast cells was obtained according to previous literature [14, 31]. Twenty μg of genomic DNA from each sample was digested with the restriction enzyme *EcoRV*, electrophoresed on a 0.8% TBE agarose gel, and then transferred to an Immobilon - Ny+ membrane (Millipore Co.), followed by a UV cross-link (BLX-E254, EEC). The fragments from the partial ORFs of the *kan* and *Asr1* genes were amplified as templates for the Southern blot probe, which was then prepared using a North2South Biotin Random Prime Labeling kit (Pierce Co., USA). The hybridization and detection were carried out according to the manufacturer's manuals

of the Chemiluminescent Nucleic Acid Detection Module (Thermo Scientific).

Ethanol Sensitivity Assay of *Asr1* Knockout Mutant and Wild-Type Strain

The differential sensitivity to ethanol was compared between the *Asr1* knockout mutant and the wild-type *S. cerevisiae* 288C. The cells were grown overnight in a YPD medium and resuspended in sterile distilled water to an optical density at 600 nm (OD_{600}) of 1.0. The cell suspensions were then serially diluted 10-fold and 3 μl spotted on plates containing 0, 8%, 10%, 11%, and 12% ethanol, followed by incubation for 2–4 days at 30°C .

The growth profiles of the wild-type *S. cerevisiae* 288C and *Asr1* knockout strain were measured as follows: yeast cells precultured in YPD medium for 24 h at 30°C were inoculated in fresh YPD medium to ensure that the different samples had the same initial cell concentration (adjusted to OD_{600} of 0.1). Next, 8% ethanol was added and the cultures were incubated at 30°C with shaking (180 rpm). The changes in absorbance of the cultures were measured every 2 h using a spectrophotometer.

Microscopy

The expression and localization of GFP-Asr1 fusion protein upon ethanol stress were performed as described previously [4], with minor modifications. Briefly, cells were grown at 30°C to an optical density at OD_{600} of 0.5, and the strains expressing the chromosomally tagged GFP-Asr1 were untreated or treated with 8% ethanol. Cells were collected at different time points, fixed in 3.7% paraformaldehyde, spread onto glass slides, and then mounted using coverslips. The nuclear DNA was stained by adding 1 $\mu\text{g}/\text{ml}$ 4',6'-diamidino-2-phenylindole (DAPI) to the cell suspension prior to stress exposure. The DAPI and GFP fluorescence were visualized using an excitation filter with a 365 nm and 450–490 nm wavelength, respectively, and then captured using a Nikon E800 with a 100 - oil immersion objective and an AxioVision imaging system, and a Nikon DS-5Mc color camera. NIS-Elements F 2.30 software was utilized for image analysis (Instruments Europe B.V., Düsseldorf, Germany) [5, 32].

RESULTS

Real-Time PCR for *Asr1* mRNA Expression Upon Ethanol Stress

Real-time PCR was used to assess the relationship between the expression level of the *Asr1* gene and ethanol stress. Using the primers shown in Table 2, PCR amplification products were obtained for the actin gene (control) and the target *Asr1* gene at 140 bp and 121 bp, respectively. In a preliminary experiment, a single dissociation peak for the product from each pair of primers suggested that the amplifications were specific and there was no primer dimer. Thus, proceeding to the next step, the expression changes of *Asr1* upon 8% ethanol stress were monitored. The results showed that the expression level of the *Asr1* gene gradually increased and reached a peak level within 2 h post stimulation. Thereafter, the expression began to

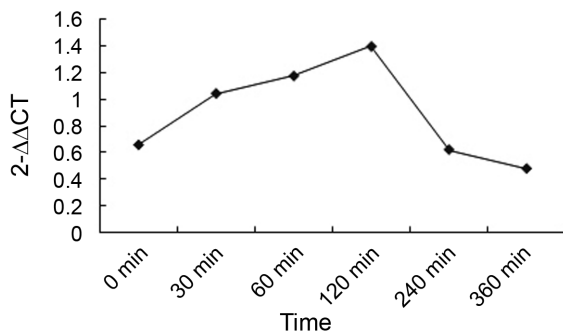


Fig. 1. Expressional changes of the *Asr1* gene upon 8% ethanol stimulation, suggesting a positive relationship between the *Asr1* gene and ethanol response in yeast.

drop, and after about 6 h it returned to the pretreatment level (Fig. 1). Therefore, the results indicate a positive relationship between the expression of *Asr1* and ethanol stress.

Targeted Disruption of *Asr1* and Construction of *Asr1*–GFP Fusion Strain

To investigate the exact biological function of the *Asr1* gene in *S. cerevisiae* 288C, the *Asr1* gene was deleted and an *Asr1*–GFP fused strain constructed using the method of homologous recombination. A 2.4-kb linear fragment from pFA6a-kanMX4 for *Asr1* deletion and a 1.5-kb GFP fusion fragment from pFA6a-GFP (S65T)–KanMX6 were obtained, as described in Materials and Methods. By transforming the fragments into competent cells of *S. cerevisiae* 288C, a DNA cassette containing the selectable marker G418 disrupted the intact *Asr1* gene. Similarly, the GFP fusion

fragment replaced the stop codon of the *Asr1* gene and was co-expressed under the control of the *Asr1* gene promoter.

Transformants were selected on YPD plates containing 350 mg/l G418. The genomic DNA was then extracted from the selected Δ *Asr1* and *Asr1*–GFP fused expression mutants, as well as from the wild-type strain *S. cerevisiae* 288C. Using the extracted genome as templates, PCR amplification using verification primers (described in Materials and Methods) and Southern blotting were both performed to validate the successful construction of the two types of mutants. In the PCR amplification, the desired 1.9 and 2.0 kb size fragments were obtained from the corresponding type of mutants (Fig. 2A and 2B). The results of Southern blot hybridization with the labeled *kan* probe showed no detectable band in the wild-type *S. cerevisiae* 288C, whereas hybridization signals were detected in the Δ *Asr1* and *Asr1*–GFP fused expression mutants (Fig. 2C). In contrast, Southern hybridization with the labeled *Asr1* probe detected hybridization signals in the wild-type strain, GFP-tagged strain, and one Δ *Asr1* mutant M7, yet no hybridized signal in the other Δ *Asr1* mutant M5 (Fig. 2D). Thus, based on the PCR and Southern hybridization results, the successful construction of the deletion strain Δ *Asr1* M5 and *Asr1*–GFP fused expression mutants was confirmed.

Change in Subcellular Localization of *Asr1p* Upon Ethanol Stimulus

To confirm whether *Asr1p* changes its subcellular localization upon ethanol stress, the localization of full-length *Asr1* fused to GFP was examined. Overnight-cultured cells grown to an optical density at OD₆₀₀ of 0.8

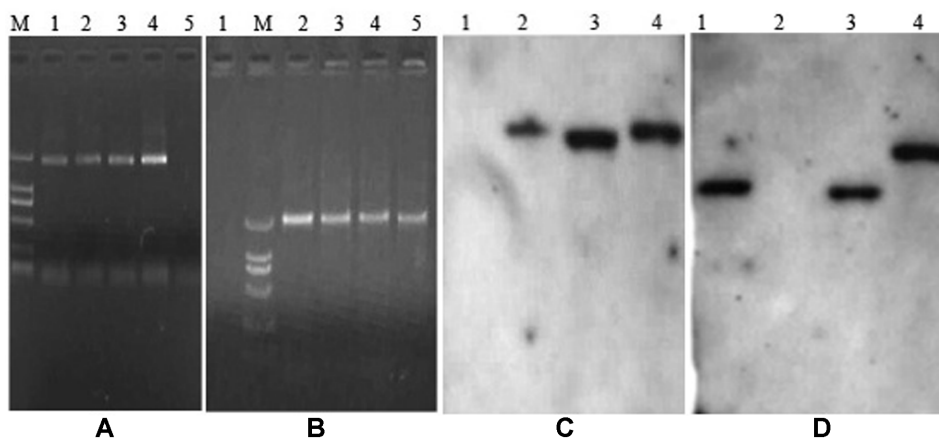


Fig. 2. PCR and Southern blot analyses of *Asr1* gene knockout and *Asr1p*–GFP fusion expression mutants.

A. PCR confirmation of Δ *Asr1* mutant strains: Lanes 1–4 are 1.9 kb PCR products from Δ *Asr1* mutants and Lane 5 is control amplification from wild-type strain S288C. **B.** PCR confirmation of *Asr1*–GFP fusion expression mutant strains: Lanes 2–5 show 2.0 kb amplified fragments from *Asr1*–GFP fusion expression mutants and Lane 1 is control amplification from wild-type strain S288C. **C, D.** Results of Southern blot to confirm successful construction of Δ *Asr1* and *Asr1*–GFP fusion expression mutants with labeled *kan* and *Asr1* probes, respectively: Lane 1 shows hybridization to wild-type genomic DNA, Lanes 2 and 3 are hybridizations to genomic DNA of Δ *Asr1* mutants M5 and M7, respectively, and Lane 4 is hybridization to genomic DNA of the *Asr1*–GFP fusion expression mutant.

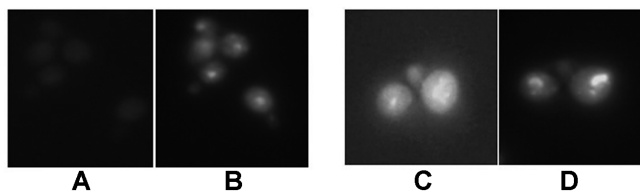


Fig. 3. Comparison of expression levels and subcellular localization of Asr1p upon ethanol exposure.

A and **B** are localization of Asr1p-GFP and nucleus stained with DAPI without ethanol stimulation, **C** shows increase in Asr1p-GFP expression and its translocation from the cytoplasm to the nucleus in the presence of 8% ethanol, and **D** shows nucleus stained with DAPI when stimulated with 8% ethanol.

were immersed in 8% ethanol for 15–30 min and the nucleus was stained with DAPI. Under normal growth conditions without alcohol, there was a slight GFP fluorescence distributed diffusely throughout the cytoplasm (Fig. 3A and 3B). However, after treatment with 8% ethanol, the fluorescence from the GFP increased significantly. Furthermore, the Asr1p-GFP was translocated from the cytoplasm and accumulated in the nucleus (Fig. 3C and 3D). Therefore, these results reconfirmed that ethanol stress led to an enhanced expression of Asr1p and impacted its subcellular localization, which is consistent with results from a previously published investigation [5].

Effect of Ethanol on Growth of *Asr1* Mutant Strain

After the successful construction of Δ *Asr1* mutants, the phenotypes of the Δ *Asr1* mutant and wild-type strains were compared in 0%, 8%, 10%, 11%, and 12% ethanol environments (Fig. 4A). The experimental results showed that, when compared with the wild-type strain *S. cerevisiae* 288C, the Δ *Asr1* mutant had a similar growth rate upon exposure to <8% ethanol. However, when the ethanol concentrations were increased to or above 8%, the Δ *Asr1* mutant was obviously more sensitive to alcohol stress than the wild-type strain. In liquid media, although the growth rates of both types of strain decreased with the addition of ethanol (data not shown), the inhibitory effect on growth was more pronounced for the Δ *Asr1* mutant than for the wild-type strain (Fig. 4B).

DISCUSSION

In the late stage of fermentation, yeast cells are commonly exposed to high concentrations of ethanol, which can damage cell membrane and perturb protein function, leading to cell growth inhibition and even death. Until now, several factors and mechanisms have been reported as being responsible for the yeast cell ethanol response, such as increases in trehalose and HSP, and changes in the gene expression and membrane composition. However,

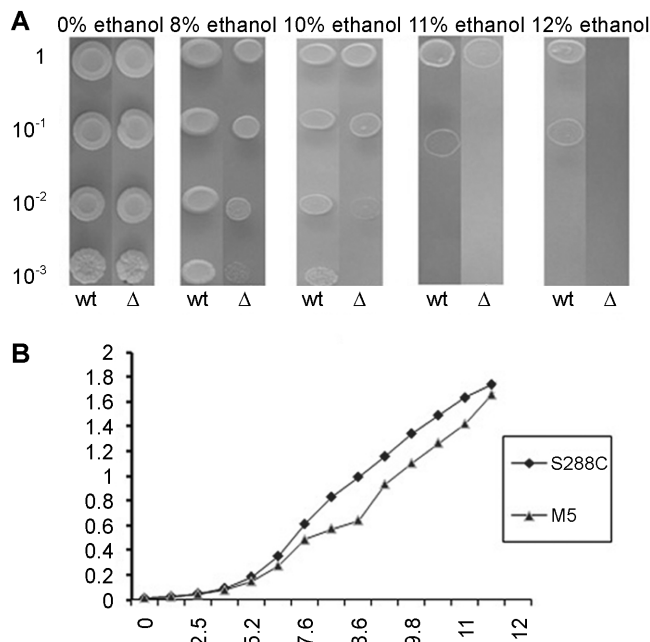


Fig. 4. Effects of ethanol on the growth of *S. cerevisiae* 288C and Δ *Asr1* mutant strain.

A. Growth of *S. cerevisiae* and Δ *Asr1* on solid YPD medium containing different concentrations of ethanol. Retarded growth was observed in *Asr1* mutants on medium with \geq 8% ethanol. **B.** Growth profiles of *S. cerevisiae* and Δ *Asr1* in liquid YPD medium containing 8% ethanol.

apart from the above-mentioned strategies, the extent of involvement of other genes or proteins in this stressful reaction and their related molecular mechanisms still need to be further elucidated.

Thus, the present study investigated the function of *Asr1* in response to elevated ethanol in *S. cerevisiae*. A relationship between Asr1p and the ethanol response was already previously reported [5], and a more recent publication by Izawa *et al.* [15] noted that Asr1p is dispensable upon alcohol exposure during sake brewing or wine-making. The present results also confirmed the involvement of Asr1p in ethanol response, where the experimental evidence showed that Asr1p was indispensable for the normal growth of yeast cells under high alcohol conditions. Real-time PCR showed that ethanol affected the expression level of *Asr1*, implying a positive relationship between *Asr1* and ethanol tolerance. The observations from fluorescence microscopy confirmed that the expression of Asr1p was accumulated and translocated into the nucleus upon ethanol stress. Furthermore, deletion of the *Asr1* gene rendered the yeast cells hypersensitive to alcohol stress.

Structural analyses have shown that Asr1p harbors two RING/PHD fingers in its N-terminal region, and RING fingers are generally considered as being responsible for protein-protein interactions and involved in a variety of

cellular processes [2, 9, 19]. Most experimental data suggests that when proteins with a RING finger domain are localized in the nucleus, they typically play an important role in controlling the chromatin structure and transcription [6, 15]. Recently, Daulny *et al.* demonstrated that the Asr1p protein is a ubiquitin-ligase that can bind directly to the RNA polymerase II *via* the carboxyl-terminal domain (CTD) and through ubiquitylation, inactivating the RNA polymerase II by ejecting the Rpb4/Rpb7 subunits from the pol II complex [10]. Nonetheless, even though such data support the hypothesis that Asr1p functions in ubiquitylation and influences the transcriptional machinery, such as pol II, there is little data supporting the relationship between Asr1p and ethanol tolerance. Consequently, the present study clearly demonstrated the involvement of Asr1p in the ethanol response. However, the complete molecular mechanism underlying the role of Asr1p in alcohol stress response remains to be defined.

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