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Role of Osmotic and Salt Stress in the Expression of Erythrose Reductase in *Candida magnoliae*

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The osmotolerant yeast, *Candida magnoliae*, which was isolated from honeycomb, produces erythritol from sugars such as fructose, glucose, and sucrose. Erythrose reductase in *C. magnoliae* (*CmER*) reduces erythrose to erythritol with concomitant oxidation of NAD(P)H. Sequence analysis of the 5'-flanking region of the *CmER* gene indicated that one putative stress response element (STRE, 5'-AGGGG-3'), found in *Saccharomyces cerevisiae*, exists 72 nucleotides upstream of the translation initiation codon. An enzyme activity assay and semiquantitative reverse transcription polymerase chain reaction revealed that the expression of *CmER* is upregulated under osmotic and salt stress conditions caused by a high concentration of sugar, KCl, and NaCl. However, *CmER* was not affected by osmotic and oxidative stress induced by sorbitol and H_2O_2, respectively. The basal transcript level of *CmER* in the presence of sucrose was higher than that in cells treated with fructose and glucose, indicating that the response of *CmER* to sugar stress is different from that of *GRE3* in *S. cerevisiae*, which expresses aldose reductase in a sugar-independent manner. It was concluded that regulation of *CmER* differs from that of other aldose reductases in *S. cerevisiae*.

Keywords: *Candida magnoliae*, erythrose reductase, aldose reductase, *Saccharomyces cerevisiae*, stress reponse

Erythritol [1,2,3,4-butanetetrol, molecular weight (MW) 122.12], a four-carbon sugar alcohol, naturally occurs in a variety of foods such as fruits, mushrooms, and fermented foods including cheese, wine, and soy sauce [6]. Owing to its excellent taste and lack of insulin-stimulating properties, erythritol has been used as a low-calorie sweetener and pharmaceutical excipient [7, 19, 25]. Most ingested erythritol are not metabolized by the human body and are excreted unchanged in the urine without altering blood glucose and insulin levels. It also prevents dental caries because the bacteria that cause dental caries are not able to utilize erythritol as a carbon source [19].

Both chemical and fermentation processes have been introduced for the large-scale production of erythritol. Erythritol can be produced from periodate-oxidized starch or dialdehyde starch by a high-temperature chemical reaction in the presence of a nickel catalyst [22, 24]. The chemical process has not been industrialized because it involves complicated steps and the starting material is expensive. Industrial production of erythritol is achieved by a fermentation process with glucose from hydrolyzed wheat and corn starches, fructose, and sucrose [2, 7]. It has been reported that osmophilic yeast and bacteria are able to produce erythritol [9, 10, 15, 26], and *Trichosporonoides megachilensis* SNG-42 has been used for commercial production of erythritol in Japan [9]. *Candida magnoliae* JH110, a yeast strain isolated from honeycomb [10], is also able to produce erythritol with high yield, and its mutant has been used to further enhance erythritol productivity [12, 23, 27].

Erythrose reductase (ER) catalyzes the final step of erythritol biosynthesis, which converts erythrose to erythritol with concomitant oxidation of NAD(P)H [13, 14, 25]. ER from a couple of microbial sources has been purified and characterized [16, 17, 25], and the genetic sequence and functional characteristics of ER from *C. magnoliae (CmER)* were recently reported [13].

In this study, the nucleotides in the 5'-flanking region were sequenced and analyzed in order to elucidate the regulatory expression of *CmER*. In addition, the effects of

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a variety of stress conditions on its expression and ER enzyme activity in *C. magnoliae* were examined.

Materials and Methods

Strains, Plasmid, and Culture Conditions

C. magnoliae JH110 [13] grown in YEPD (1% yeast extract, 2% peptone, 2% glucose) was stored at −80°C. *Escherichia coli* TOP10 [F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lacZ*ΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*) 7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G] (Invitrogen, Carlsbad, CA, USA) was used for plasmid DNA preparation and was routinely grown in LB medium (0.5% yeast extract, 1% peptone, and 1% NaCl) at 37°C. LB plates supplemented with 50 mg/l ampicillin were used to select for plasmid-harboring *E. coli*. The pGEM-T plasmid (Promega, Madison, WI, USA) was used for DNA walking.

For the ER enzyme activity assay and reverse transcription polymerase chain reaction (RT–PCR) analysis, *C. magnoliae* was grown in YEPD medium at 30°C to an optical density (OD_{600}) of 1.0, harvested by filtration to remove medium, transferred to YEPD supplemented with stress inducers for 1 h, and then collected by filtration. Sugars (fructose, glucose, and sucrose), KCl, NaCl, methylglyoxal, H_2O_2, and sorbitol at high concentrations were used as stress inducers. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

DNA Walking and Sequence Analysis

The genomic DNA sequence in the 5'-upstream region (promoter region) of the *CmER* gene (GenBank No. FJ550210) was identified by genomic walking, which was performed with the DNA Walking SpeedUp Kit (Seegene, Seoul, Korea) according the manufacturer's protocol. Promoter analysis of the *CmER* gene was performed using the Web-based promoter database of *Saccharomyces cerevisiae* (SCPD, http://rulai.cshl.edu/SCPD/).

Preparation of Cell Extracts

Harvested cells were broken by vortexing with acid-washed glass beads (Sigma-Aldrich) in 1 ml of 50 mM phosphate buffer (pH 6.5) for 3 min. Cell homogenates were centrifuged at 15,000 ×*g* for 20 min at 4°C, and the supernatants were used as cell extracts. Cell extracts were prepared from three independent cultures. Protein concentrations were determined by the Bio-Rad assay using bovine serum albumin as the standard.

ER Enzyme Activity Assay

The ER assay mixture (250 μl) contained crude enzyme, 12 mM D-erythrose, and 4 mM NADPH in 50 mM phosphate buffer (pH 6.5). ER activity was determined by measuring the decrease in OD_{340} at 37°C for 10 min. One unit of ER activity was defined as the amount of enzyme catalyzing the oxidation of 1 μmol of NADPH per minute at 37°C. Specific ER activity was expressed as units per milligram of cellular protein [13].

RNA Isolation and RT–PCR

Total RNA was prepared using TRIzol reagent (Invitrogen). RT–PCR was performed using the cDNA EcoDry Premix (Takara Bio, Shiga, Japan) according to the manufacturer's instructions. Total RNA (0.5 μg) and primers CmER-F (5'-ATGTCTTCGACCTACACCC-3') and CmER-R (5'-CGTCGGCGTACTTCATGT-3') were used in 30 cycles of RT–PCR amplification. Aliquots of the PCR product were separated on a 0.8% agarose gel.

Results and Discussion

Sequence Analysis of the 5'-Flanking Region

A 450 bp genomic DNA sequence containing the 5'-upstream flanking region of the *CmER* gene was identified, aligned, and compared with the promoter sequences of the stress-responsive genes in *S. cerevisiae* (Fig. 1). As with other stress-controlled genes in *S. cerevisiae* such as *CTT1* or *HSP104*, one putative stress-response element (STRE) sequence, 5'-AGGGG-3' [11], was found 72 nucleotides upstream of the initiation codon, indicating that *CmER* might be regulated in a similar manner to the *GRE3* gene in *S. cerevisiae*, which has one STRE 144 nucleotides upstream of the translation initiation codon.

Although the exact biological roles of yeast aldo-keto reductases (AKRs) are largely unknown, it has been suggested that AKRs such as Gre3, Gcy1, and Ypr1 play crucial roles in the stress response of the budding yeast *S. cerevisiae* [4, 20, 21]. *ScGRE3* is upregulated by a wide variety of stress conditions including osmotic stress, heat-shock, and oxidative stress [4]. Transcription of *ScGRE3* is positively regulated by Msn2 and Msn4, two well-known stress-responsive transcription factors in *S. cerevisiae* [5].

C.magnoliae	*ER*	-72	T T A G G G G A	-65
	ALD4	-194	T G A G G G G C	-187
		-317	A A A G G G G G	-310
	ALD6	-251	T G A G G G G G	-244
	CTT1	-380	C A A G G G G A	-373
		-365	T A A G G G G C	-358
	DDR2	-251	T T A G G G G A	-244
		-206	A A A G G G G A	-199
	GCY1	-286	A A A G G G G T	-279
	GPH1	-359	T A A G G G G T	-352
S.cerevisiae		-328	T C A G G G G A	-321
	GRE3	-144	C T A G G G G C	-137
	HSP12	-437	T A A G G G G A	-430
		-416	G C A G G G G G	-409
		-379	A G A G G G G A	-372
	HSP104	-254	T A A G G G G C	-247
		-222	A C A G G G G G	-215
		-274	A A A G G G G C	-267
	TPS2	-310	A T A G G G G T	-303
	Consensus		A G G G G	

Fig. 1. Alignment of STRE-related sequences in the 5'-flanking region of stress-inducible genes in *C. magnoliae* and *S. cerevisiae*. Conserved sequences are shaded in black. Numbers indicate nucleotides upstream of the translation initiation (ATG) codon of each gene.

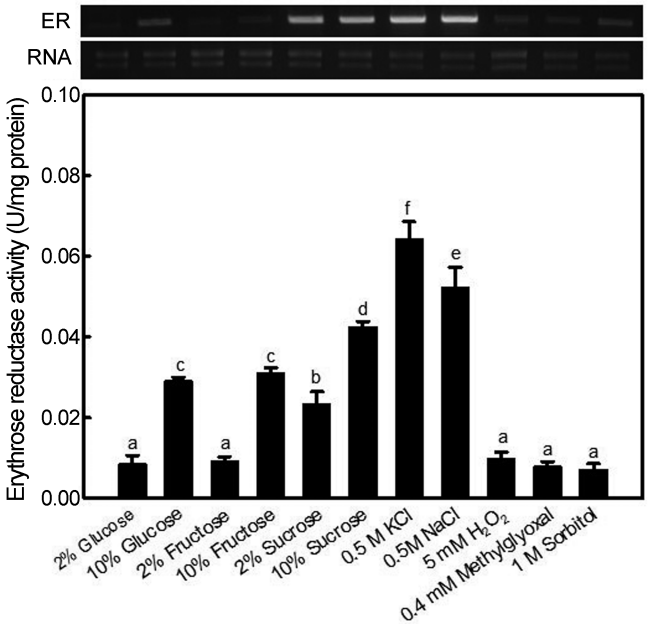

Fig. 2. Level of erythrose reductase transcripts and enzyme activity under a variety of stress conditions.
Total RNA (0.5 μg) was used for a semiquantitative RT–PCR reaction comprised of 30 cycles. PCR products were separated on a 0.8% agarose gel, and ethidium-bromide-stained rRNA for each sample is shown as the loading control. Enzyme assay results were obtained from three independent experiments, and averages and standard errors are shown. Based on Duncan's Multiple Range Test ($p<0.05$), a different letter indicates a significant difference between means.

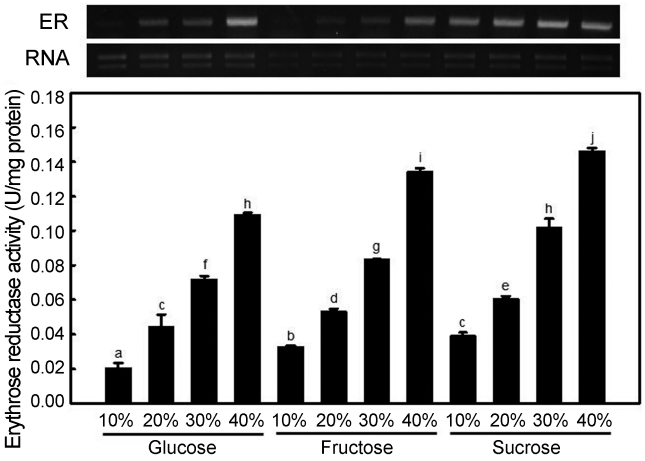

Fig. 3. Erythrose reductase transcript level and enzyme activity in the presence of fructose, glucose, and sucrose.
Procedures for the enzyme activity assay and RT–PCR are the same as those in Fig. 2. Duncan's Multiple Range Test ($p<0.05$), a different letter indicates a significant difference between means.

Regulatory Expression of the *CmER* Gene Under Stress Conditions

Expression of the *CmER* gene was examined in the presence of different carbon sources. In addition, the effects of a variety of stress conditions induced by NaCl, KCl, sorbitol, H_2O_2, and methylglyoxal on gene transcription and ER enzyme activity were investigated.

As concentrations of fructose and glucose increased, ER activity was notably enhanced (Fig. 2). ER enzyme activity was enhanced more than 2-fold as the sucrose concentration increased from 2% to 10% without a drastic change in transcript level. The basal transcript level of ER in the presence of 2% sucrose was higher than that observed after treatment with 2% glucose or 2% fructose, indicating that expression of the *CmER* gene might be carbon-source-dependent. Based on the analysis of ER transcript level in the presence of 2% glucose and 2% sucrose, ER expression was seemingly induced by sucrose. In addition, the transcript level after treatment with 10% fructose was notably lower than the level observed for 2% sucrose, although the former gave rise to an elevated ER enzyme activity. Accordingly, it was speculated that a peculiar regulatory circuit may exist, and the response of *CmER* under different stress conditions should be considered at both the transcriptional and post-transcriptional levels. The ER enzyme activities in the presence of glucose, fructose, and sucrose observed in this study were consistent with previous reports that higher erythritol productivity results when fructose or sucrose is used as a carbon source instead of glucose [10]. Notably, the carbon source-dependent expression of *CmER* differed from *ScGRE3* expression, which is carbon-source-independent [1].

A significant increase in the transcript level of ER was also observed when the cells were grown under stress conditions induced by KCl and NaCl, indicating that salt stress as well as sugar stress is involved in transcription of the *CmER* gene. Treatment with 0.5 M KCl exerted a profound effect on both the transcript level and ER enzyme activity, which may support the finding that the erythritol production rate is significantly enhanced by supplementation of KCl into the cultivation medium [3, 18].

Since both enzyme activity and transcript level were enhanced in the presence of 0.5 M KCl and 0.5 M NaCl, it was thought that the *CmER* gene is upregulated under salt stress. However, neither transcript level nor enzyme activity was significantly affected by oxidative stress caused by H_2O_2 or 1.0 M sorbitol. It should be mentioned that *CmER* expression was not affected by oxidative stress induced by H_2O_2, which is not the case for *ScGRE3* expression [1]. Treatment of the cells with methylglyoxal did not result in an increase in ER enzyme activity.

The ER enzyme activity and transcript level were further examined in the presence of a variety of sugar concentrations. As shown in Fig. 3, the ER enzyme activity was closely related to sugar concentration; that is the increase in ER enzyme activity with increased sugar concentration was nearly linear. The highest ER enzyme activity was obtained at 40% sucrose, which is partially

consistent with the results shown in Fig. 2. A clear increase in the ER transcript level was observed with increases in the concentrations of fructose and glucose, although there were some different transcription patterns between fructose and glucose. When the cells were grown in fructose, the increase in ER transcription level was not as significant as that in glucose.

The results of this study revealed that *CmER* transcription is upregulated in order to increase the ER enzyme activity under osmotic and salt stress conditions. The upregulation of *CmER* transcription under osmotic and salt stress conditions was consistent with the expression trends of *GRE3* in *S. cerevisiae*. However, unlike *ScGRE3*, the expression of *CmER* was found to be carbon-source-dependent in this study. Moreover, *CmER* did not respond to oxidative stress induced by H_2O_2.

This is the first report of the regulatory expression of erythrose reductase in *C. magnoliae*. Future studies should explore the genetic circuit underlying the stress response in *C. magnoliae*.

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