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GENETICS AND MOLECULAR BIOLOGY OF INDUSTRIAL ORGANISMS

Decreased fluidity of cell membranes causes a metal ion deficiency in recombinant *Saccharomyces cerevisiae* producing carotenoids

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Abstract The genome-wide transcriptional responses of *S. cerevisiae* to heterologous carotenoid biosynthesis were investigated using DNA microarray analysis. The results show that the genes involved in metal ion transport were specifically up-regulated in the recombinant strain, and metal ions, including Cu^{2+}, Fe^{2+}, Mn^{2+}, and Mg^{2+}, were deficient in the recombinant strain compared to the ion content of the parent strain. The decrease in metal ions was ascribed to a decrease in cell membrane (CM) fluidity caused by lower levels of unsaturated fatty acids and ergosterol. This was confirmed by the observation that metal ion levels were restored when CM fluidity was increased by supplying linoleic acid. In addition, a 24.3 % increase in the β-carotene concentration was observed. Collectively, our results suggest that heterologous production of carotenoids in *S. cerevisiae* can induce cellular stress by rigidifying the CM, which can lead to a deficiency in metal ions. Due to the importance of CM fluidity in cellular physiology, maintaining normal CM fluidity might be a potential approach to improving carotenoid production in genetically engineered *S. cerevisiae*.

Keywords *Saccharomyces cerevisiae* · Heterologous carotenoid biosynthesis · DNA microarray · Cell membrane fluidity · Metal ions deficiency

Introduction

Carotenoids are a diverse class of C40 isoprenoids with multiple physiological and nutritional roles in plants, algae, bacteria, and fungi. In humans, β-carotene is the precursor for vitamin A, may function as an anti-oxidant, has protective properties against cancer and stimulates the immune system [1]. Microbial sources of carotenoids have received increasing attention because of the restricted rules and regulations currently applied to obtaining chemicals. In recent years, carotenoids have been successfully synthesized in non-carotenogenic organisms such as *Escherichia coli* and *Saccharomyces cerevisiae*, and this method of production has been extensively investigated as a metabolic engineering strategy [2, 3]. In contrast to other hosts, *S. cerevisiae* exhibits efficient isoprenoid metabolism and is capable of accumulating large quantities of the triterpenoid ergosterol in its membranes. Furthermore, *S. cerevisiae* strains are generally regarded as safe (GRAS) organisms. These properties make *S. cerevisiae* an excellent candidate for the production of β-carotene for pharmaceutical, nutritional and feed applications [2]. In *S. cerevisiae*, β-carotene is synthesized via the mevalonate pathway (MVA pathway). In this pathway, IPP is isomerized to DMAPP and converted to GGPP via the enzyme GGPP synthase. The co-expression of three exogenous genes (and the production of their protein products), *crtE* (GGPP synthase), *crtYB* (encods bifunctional phytoene synthase and lycopene cyclase) and *crtI* (phytoene desaturase), drives two GGPP molecules into the carotenoid pathway, which synthesizes

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the orange-colored β-carotene molecule through condensation, dehydrogenation and two cyclization reactions (Fig. S1) [2, 4].

A variety of metabolic strategies have been utilized to significantly improve carotenoid production in *E. coli* and *S. cerevisiae*. These efforts have involved the optimization of native pathways, introduction of foreign genes to enhance metabolic flux, and balancing of co-factors [2, 5, 6]. However, the metabolic pathway of heterologous bio-compound production is not an isolated process. Genetically engineering an organism to accumulate high levels of a heterologous product can have a global effect on the metabolism of the organism and can cause a metabolic burden, an overconsumption of resources, or the release of toxic intermediates [7]. This phenomenon is important in the heterologous production of carotenoids in *S. cerevisiae* because the mevalonate pathway provides many functional compounds, including sterols, dolichols and ubiquinones. Heterologous carotenoid formation can decrease the levels of these compounds through competition for precursors, such as FPP, which in turn can negatively influence cell growth and carotenoid production [8].

When a cell faces an alteration of its physiological state caused by heterologous bio-compound biosynthesis, it will re-program its genomic expression as an adaptive response [9]. Hence, revealing the transcriptional and physiological changes in strains producing carotenoids can help us to understand the mechanisms of how these cells adapt to carotenoid accumulation. This can also help us to identify the potential factors limiting the formation of carotenoids and design effective strategies to improve carotenoid production [10, 11]. DNA microarray is a powerful tool to achieve these goals. Verwaal et al. [11] used DNA microarray to study the transcriptome of two laboratory *S. cerevisiae* strains (CEN.PK113-7D) that produce different levels of carotenoids in carbon-limited chemostat cultures. They found that multidrug-resistant transporter synthesis, encoded by pleiotropic drug-resistance genes (*PDR5*, *PDR10* and *PDR15*), was induced. This facilitated the secretion of carotenoids to the environment to decrease the toxicity within the cells [11]. It has recently been shown that the carotenoid hyper-producer *S. cerevisiae* strain (strain GSY1136 derived from laboratory strain FY2) undergoes adaptive evolution with periodic hydrogen peroxide shocking, resulting in the up-regulation of genes related to lipid biosynthesis pathways [12].

To further elucidate the transcriptional and physiological responses of *S. cerevisiae* cells to heterologous carotenoid biosynthesis, a *S. cerevisiae* strain used in the wine industry (T73-4) was used to construct recombinant *S. cerevisiae* strains that produce β-carotene [13]. DNA microarray was used to investigate the transcriptional and physiological discrepancies of the recombinant strains relative to the parent strain. The results revealed that metal ions in recombinant strains, including Cu^{2+}, Fe^{2+}, Mn^{2+}, and Mg^{2+}, became deficient compared to the levels in the parent strain. This was ascribed to the decreased fluidity of the cell membrane (CM) caused by a reduction in the UFA and ergosterol contents of the cells.

Materials and methods

Microorganism strains and medium

The recombinant *S. cerevisiae* T73-H was constructed by the integration of YIplac211YB/I/E* (kindly provided by Dr. Rene Verwaal from the Netherlands) [2] containing *crtYB, crtI* and *crtE* from *Xanthophyllomyces dendrorhous* into yeast strain T73-4* (kindly provided by Professor Marcel li del Olmo Muñoz from Spain) [14]. The expressions of three genes were driven by the strong constitutive *S. cerevisiae* GPD promoter and the CYC1 terminator. Production of the parent strain T73-W was achieved by the integration of YIplac211 into the T73-4 strain. A yeast nitrogen base (YNB) medium (0.67 % amino acid-free Difco yeast nitrogen base and 2 % glucose) was used in the study.

Culture conditions

Yeast strains were aerobically pre-cultured in YPD medium (2 % glucose, 2 % peptone, and 1 % yeast extract) and agitated at 200 rpm for approximately 15 h until reaching the late-exponential phase of growth. The initial yeast inoculum consisted of 5×10^5 CFU/mL in 500 mL flasks containing 200 mL YNB medium that was continuously agitated 180 rpm at 30° C. Samples were taken at the indicated time points and analyzed for cell growth and for ergosterol, fatty acid, β-carotene and total carotenoid content. To test the effects of metal ion addition, different concentrations of $CuCl_2$ (50, 500, and 2 mM), $FeCl_2$ (50, 500, and 2 mM), $MnSO_4$ (50, 500, 2, and 10 mM), $CaCl_2$ (100, 1, and 4 mM), $ZnSO_4$ (50, 500, and 1 mM) and $MgCl_2$ (100, 1, 4, and 10 mM) were added to the initial medium. The samples were cultivated for 22 h and collected to determine β-carotene content. All experiments were independently repeated three times, and data in the figures and table are expressed as averages ± standard deviations.

Analytical methods

Based on the methods of Oh et al. [15] and Shang et al. [16], dry cell weight (DCW) was initially determined gravimetrically and showed a functional relationship to the spectrophotometrically measured optical density at 660 nm (DU-70 Beckman Inc., Fullerton, CA). Therefore, in this

study, DCW concentration was calculated from $OD_{660\ nm}$ values using the following equation: Dry cell weight (g/L) = 0.49 × OD_{660nm}. Carotenoids were extracted from the cultured cells with acetone-methylene chloride (50:50, *v*/*v*) and analyzed with high-performance liquid chromatography (HPLC). HPLC separation and quantization were performed in a Symmetry C18 column (4.6 × 150 mm) eluted isocratically with an acetonitrile:methanol:2-propanol mixture (85:10:5, *v*/*v*/*v*) at a flow rate of 1 mL/min. The separated carotenoids were detected at 450 nm, and the spectra were recorded online. For quantification, a standard calibration curve based on at least seven solutions of known β-carotene concentration (Sigma-Aldrich) was constructed. The content of the β-carotene was calculated as the milligrams per extracted gram dry cell weight (DCW).

Ergosterol content was measured as described by Shang et al. [16]. Fatty acids from whole yeast cells were extracted and methyl-esterified as described by Zhang et al. [17]. Fatty acid methyl esters were analyzed by GC–MS (ISO, Thermo, USA) in a Thermo Scientific TRACE TR-WaxMS capillary GC column (30 m × 0.32 mM × 0.25 μM) with a helium carrier gas (1 mL/min). The lipid standards (fatty acid methyl ester mixtures) were purchased from Wako Chemicals or from Sigma. Total fatty acid concentrations were calculated as the sum of C8–C22 (saturated and unsaturated). The ergosterol and fatty acid contents were expressed as the mg/g dry cell weight.

Microarray procedures

For the DNA microarray analysis, late-exponential-phase cells of each strain were harvested by centrifugation and stored at −80° C until RNA isolation. Three independent cultures were prepared for biological repeats. A Yeast Genome 2.0 Array was obtained from CapitalBio Corporation (Beijing, China). Total RNA was extracted by a hot acidic phenol method [18]. The purity and integrity of the RNA was confirmed by agarose gel electrophoresis. For cDNA synthesis and for cRNA synthesis and labeling, a One-Cycle Target Labeling kit and control reagents (Affymetrix) were used according to the manufacturer's instructions. Biotinylated cRNA was fragmented and used as a probe. The probe was hybridized to the DNA microarray in a CapitalBio BioMixer™ II Hybridization Station overnight at 45 °C for 18 h and washed with two consecutive solutions. Arrays were scanned using a confocal LuxScanTM (CapitalBio Corp., China) scanner and data were extracted with LuxScanTM 3.0 software (CapitalBio Corp., China). LOWESS normalization was performed on the data [19]. For data extraction, spots with signal intensities below 800 units, after subtracting the background, were removed from both dye channels (Cy3 and Cy5). A significant analysis of the microarray (SAM) was applied to identify the genes differentially expressed between the recombinant strain T73-H and the parent strain T73-W [20]. The threshold for significance was set to allow a median of one false positive per analysis, for a false discovery rate (FDR) of <0.05 %. Genes whose expression levels were greater than twofold or less than 0.5-fold, relative to the parent strain, were considered to be induced or repressed, respectively. The complete list of all differentially expressed genes is provided in the supplementary Table S2. The affected genes were further categorized by biological process and function using the *Saccharomyces* Genome Database Gene Ontology Term Finder tools (*SGD* GO Term Finder http://www.yeastgenome.org/cgi-bin/GO/goTermFinder.pl) (*p*-values <0.05 were considered significant) and the related data can be found in Table S3.

Evaluation of membrane fluidity

Cell membrane (CM) fluidity was evaluated by measuring the fluorescence anisotropy of 1,6-diphenyl-1,3,5-hexatriene (DPH). As described by Sharma [21], harvested cells were suspended in a 0.1 M potassium phosphate buffer (pH 6.8) to a density of 500 mg dry cell weight mL^{-1}. The mixtures were incubated in a thermostatically controlled and darkened chamber at 25 °C for 40 min with DPH at a final concentration of 0.5 mM. After incubation, cells were washed three times with the same buffer. Fluorescence anisotropy was measured using a spectrofluorometer (model 650-60; Hitachi, Tokyo, Japan) with 360 and 450 nm as the excitation and emission wavelengths, respectively. Fluorescence anisotropy (Υ) was calculated as follows:

$$r = (I_{VV} - GI_{VH})/(I_{VV} + GI_{VH}),\ G = I_{VH}/I_{HH}$$

where I_{VV} and I_{VH} indicate the values of the fluorescence intensities determined at the vertical and horizontal orientations of the emission polarizer when the excitation polarizer is set in the vertical position. A similar equation was used, but with the variables I_{HV} and I_{HH}, when the excitation polarizer was set in a horizontal position. *G* is a correction factor for background fluorescence and light scattering. Decreases in the degree of fluorescence anisotropy represent increases in the fluidity of the lipid bilayer, which controls or alters the mobility of DPH in the membrane.

ICP-OES analysis of metal ions

Cells grown to the late-exponential phase were harvested by centrifugation for 10 min at 10,000×*g*. The cells were washed twice in HPLC-grade water, transferred to prepared tubes and covered with a cap. The cells were digested using concentrated nitric acid at 80 °C for 1 h. Water was added to the digested cells to a final volume of 1 mL. Total copper, iron, manganese, magnesium, calcium and zinc in the

samples were determined by Thermo iCAP 6000 inductively coupled plasma-optical emission spectrometry (ICP-OES) analysis (Thermo Fisher Scientific, USA). The concentrations of metal ions were expressed as $\mu g/OD_{660}$.

Results and discussion

Cell growth and carotenoid production of the recombinant *S. cerevisiae* strain

Figure 1 shows the profiles of cell growth (A), glucose consumption of both strains and β-carotene synthesis in the recombinant strain T73-H (B). Cell growth (OD_{660} values) of the recombinant strain T73-H was inhibited due to a metabolic burden. The strain reached the late-exponential phase after 16 h of fermentation, which was approximately 2 h later than the parent strain T73-W reached this stage. Correspondingly, the highest biomass (OD_{660} values) of the recombinant strain was decreased by 49.8 % compared to biomass of the T73-W strain. The consumption of glucose also decreased. However, after 14 h of fermentation, there were no significant differences between the strains. β-carotene production was not found in the parent strain due to the lack of carotenogenic genes. The β-carotene contents in the recombinant strain gradually increased as fermentation proceeded, and the highest β-carotene content (4.02 mg/g DCW) was achieved at 22 h.

DNA microarray

The specific rate of bio-compound production represents the production capacity of the cells. Therefore, we used samples from the late-exponential phase of the recombinant strain (cultivated for 16 h) for DNA microarray analysis because the highest specific carotenoid production rates (0.33 mg/h/g DCW) were achieved at this phase. Cells of the parent strain from samples cultivated for 14 h were selected as reference samples. Microarray data analysis revealed that there were 140 and 202 genes that were up-regulated (greater than twofold expression) and down-regulated (less than 0.5-fold expression), respectively, in T73-H relative to the expression in T73-W (Table S2). The induced genes were further categorized according to their biological and functional process as assigned by the *Saccharomyces* Genome Database (*SGD*) (Table S3). The biological processes associated with the up-regulated genes were related to "ion transport", "iron ion transport", "copper ion transport", "carboxylic acid transport" and "organic acid transport", while the processes associated with the down-regulated genes included "ribonucleoprotein complex biogenesis", "ribosome biogenesis", "ncRNA processing" and "RNA processing". The repression of ribosomal genes is a general feature of the environmental stress response (ESR) [22]. It has been demonstrated that ESR genes are down-regulated in response to environmental challenges, such as cold, oxygen deprivation and respiratory inhibition, and result in a decrease in growth rates [23, 24]. The down-regulation of ESR genes in this study suggests that heterologous carotenoid biosynthesis in *S. cerevisiae* can cause stressful conditions for yeast cells and can result in decreased cell growth (Fig. 2), which is consistent with the conclusion of Verwaal et al. [11].

Because up-regulated genes can reflect functional changes in a cell in response to heterologous compound biosynthesis [25], we focused on the group of up-regulated genes in the discussion below. The genes induced (twofold or more) in T73-H vs T73-W were categorized according to

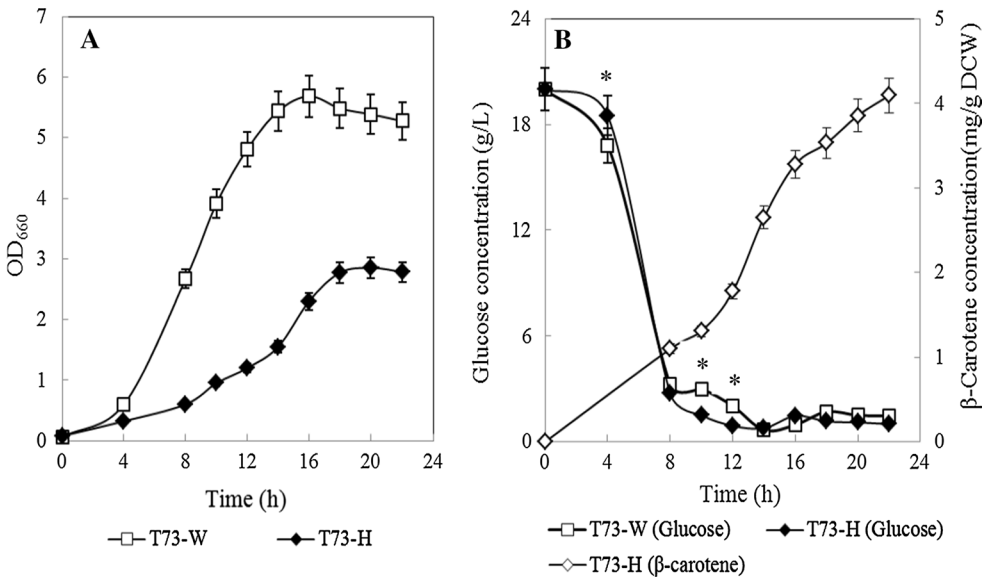

Fig. 1 Cell growth (**a**), glucose consumption and β-carotene concentrations (**b**) of the parent strain, T73-W, and the recombinant strain, T73-H. Values are the mean ± standard deviations of three experiments. The *asterisks* indicate a significant difference between the data obtained from strain T73-W and from T73-H ($p < 0.05$, Student's *t* test)

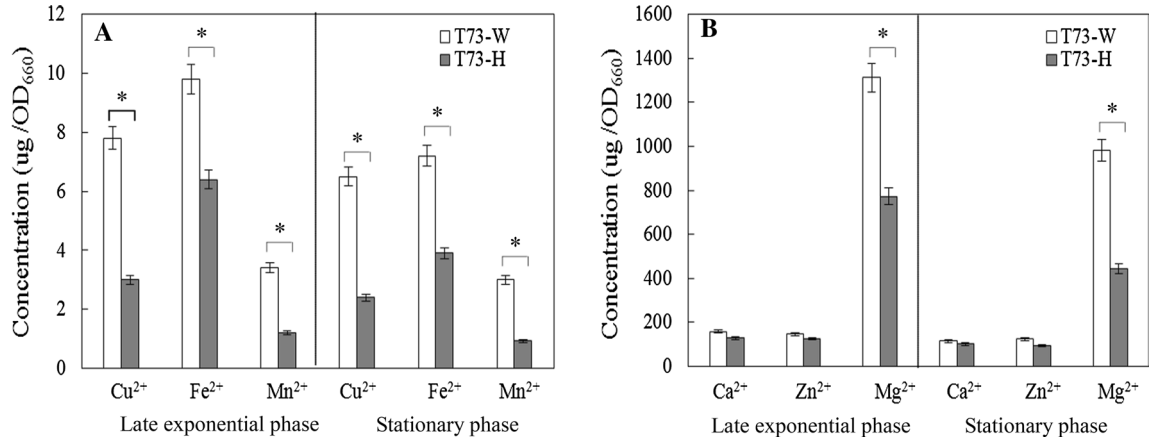

Fig. 2 Concentrations of Cu^{2+}, Fe^{2+}, Mn^{2+} (**a**) and Ca^{2+}, Zn^{2+}, Mg^{2+} (**b**) in the parent strain, T73-W, and the recombinant strain, T73-H, at the late-exponential and stationary phases. Values are the mean ± standard deviations of three experiments. The *asterisks* indicate a significant difference between the data obtained from strain T73-W and from T73-H ($p < 0.05$, Student's *t* test)

their biological process (Table 1). The differences in genes from T73-H vs T73-W that are involved in the processes of "ion transport", "iron ion transport", "copper ion transport" had the lowest *P* values, strongly suggesting that the genes in the functional categories related to metal ion metabolism are induced as a response to carotenoid accumulation. The "ion transport" category genes included *FRE2*, *FRE7*, *CTR3*, *MRS3,* and *FRE1*. Genes in "iron ion transport" category included *FRE2*, *FRE7*, *MRS3*, and *FRE1,* while the group of "copper ion transport" genes contained *FRE2*, *FRE7*, *CTR3*, and *FRE1*. It is known that *CTR3* encodes the high-affinity Cu^{2+} transporter and a Mn^{2+} transporter, which are active in metal-limited cells [26]. *FRE1*, *FRE2* and *FRE7* encode the Fe^{3+}/Cu^{2+} reductase involved in iron metabolism, and their mRNA levels are increased by iron-limited growth [27]. *MRS3,* an iron transporter, mediates Fe^{2+} transport across the inner mitochondrial membrane and is active under low-iron conditions [28]. *ATX2,* which encodes the high-affinity Mn^{2+} transporter, was also up-regulated (Table S2) and is induced in metal-limited cells [29]. As previously shown by Verwaal et al. [11], *PDR5*, which encodes the plasma membrane ABC-transporter involved in cellular detoxification, was up-regulated in the present study (Table S2). This supports previous suggestions that carotenoids can cause membrane stress and that these transporters might bind, remove and secrete carotenoids from membranes [11]. It should be mentioned that there was very little overlap in the genes categories between the results of Verwaal et al. [11] and our results. This might be due to the different methods of cultivation used between the two studies. In the former study, a carbon-limited chemostat culture was used, which strictly controls the growth conditions and maintains a constant and specific growth rate, with uniform concentrations of all metabolites and substrates. In the present study, we used batch cultivation in which the metabolite contents were continuously changing. According to previous research, different methods of cultivations can result in distinct transcriptome profiles [30].

Deficiency of metal ions in the recombinant strain

In *S. cerevisiae*, increasing the mRNA transcript levels of metal ion transport genes is one strategy to respond to metal ion deficiencies in cells [27]. The results obtained here led to an assumption that metal ions became limited in carotenoid-producing yeast. It has been suggested that some metal ions, such as Cu^{2+}, Fe^{2+}, Mn^{2+}, Ca^{2+}, Mg^{2+}, and Zn^{2+}, are essential for cell growth and microbial carotenoid biosynthesis [31]. Therefore, we suspected that these metal ions might be limited in yeast cells and be a limiting factor in β-carotene production. To confirm this prediction, the intracellular concentrations of metal ions in both yeast strains were determined at times corresponding to the late-exponential phase and the stationary phase. Concentrations of Cu^{2+}, Fe^{2+}, Mn^{2+}, Ca^{2+}, Mg^{2+}, and Zn^{2+} were measured (Fig. 2). The data show that the concentrations of these ions in the recombinant strain were significantly decreased compared to those of the parent strain, T73-W. The concentrations of Mg^{2+}, Cu^{2+}, Fe^{2+}, and Mn^{2+} in the T73-H strain were only 58.9, 38.2, 65.8, and 36.3 %, respectively, of that in the T73-W strain at the late-exponential phase. At the stationary phase, the concentrations in the T73-H strain were 45.3, 36.4, 54.8, and 30.9 %, respectively, of the parent strain. No significant differences were observed in Ca^{2+} or Zn^{2+} concentrations. These results confirmed that metal ions were deficient in recombinant yeast cells.

Transition metals are important components of living cells and play diverse roles in cellular processes. Therefore,

Table 1 Genes up-regulated in T73-H vs T73-W categorized by biological process (twofold or more)

Systematic name	Standard name	Fold	Description[a]
Ion transport			
YKL187C	*FAT3*	7.6919	Fatty acid transporter
YOL158C	*ENB1*	4.9693	Endosomal ferric enterobactin transporter
YOR348C	*PUT4*	4.8528	Proline permease
YKR039W	***GAP1***	4.5568	General amino acid permease
YCL025C	***AGP1***	4.1264	Low-affinity amino acid permease
YPL265W	*DIP5*	3.5648	Dicarboxylic amino acid permease
YKL220C	***FRE2***	2.9971	Ferric reductase and cupric reductase
YOL152W	***FRE7***	2.7469	Putative ferric reductase
YKL188C	*PXA2*	2.5136	Subunit of a heterodimeric peroxisomal ATP-binding cassette transporter complex
YLR411W	***CTR3***	2.461	High-affinity copper transporter of the plasma membrane
YJL133W	***MRS3***	2.4555	Iron transporter
YHL016C	*DUR3*	2.1843	Plasma membrane transporter for both urea and polyamines
YOR273C	*TPO4*	2.1783	Polyamine transport protein
YCR075C	*ERS1*	2.1436	H(+)-driven transporter involved in L-cystine export
YGR260W	*TNA1*	2.0724	High-affinity nicotinic acid plasma membrane permease
YLR214W	***FRE1***	2.0382	Ferric reductase and cupric reductase
YOR137C	*SIA1*	2.0378	Protein of unassigned function
Iron ion transport			
YOL158C	*ENB1*	4.9693	Endosomal ferric enterobactin transporter
YKL220C	***FRE2***	2.9971	Ferric reductase and cupric reductase
YOL152W	***FRE7***	2.7469	Putative ferric reductase
YJL133W	***MRS3***	2.4555	Iron transporter
YLR214W	***FRE1***	2.0382	Ferric reductase and cupric reductase
Copper ion import			
YKL220C	***FRE2***	2.9971	Ferric reductase and cupric reductase
YOL152W	***FRE7***	2.7469	Putative ferric reductase
YLR411W	***CTR3***	2.461	High-affinity copper transporter of the plasma membrane
YLR214W	***FRE1***	2.0382	Ferric reductase and cupric reductase
Carboxylic acid transport			
YKL187C	*FAT3*	7.6919	Fatty acid transporter
YOR348C	*PUT4*	4.8528	Proline permease
YKR039 W	***GAP1***	4.5568	General amino acid permease
YCL025C	***AGP1***	4.1264	Low-affinity amino acid permease
YPL265 W	*DIP5*	3.5648	Dicarboxylic amino acid permease
YKL188C	*PXA2*	2.5136	Subunit of a heterodimeric peroxisomal ATP-binding cassette transporter complex (Pxa1p-Pxa2p)
YCR075C	*ERS1*	2.1436	H(+)-driven transporter involved in L-cystine export
YGR260 W	*TNA1*	2.0724	High-affinity nicotinic acid plasma membrane permease
Organic acid transport			
YKL187C	*FAT3*	7.6919	Fatty acid transporter
YOR348C	*PUT4*	4.8528	Proline permease
YKR039 W	***GAP1***	4.5568	General amino acid permease
YCL025C	***AGP1***	4.1264	Low-affinity amino acid permease
YPL265 W	*DIP5*	3.5648	Dicarboxylic amino acid permease
YKL188C	*PXA2*	2.5136	Subunit of a heterodimeric peroxisomal ATP-binding cassette transporter complex (Pxa1p-Pxa2p)
YCR075C	*ERS1*	2.1436	H(+)-driven transporter involved in L-cystine export
YGR260 W	*TNA1*	2.0724	High-affinity nicotinic acid plasma membrane permease

[a] Descriptions according to those of *Saccharomyces* Genome Database (http://www.yeastgenome.org)

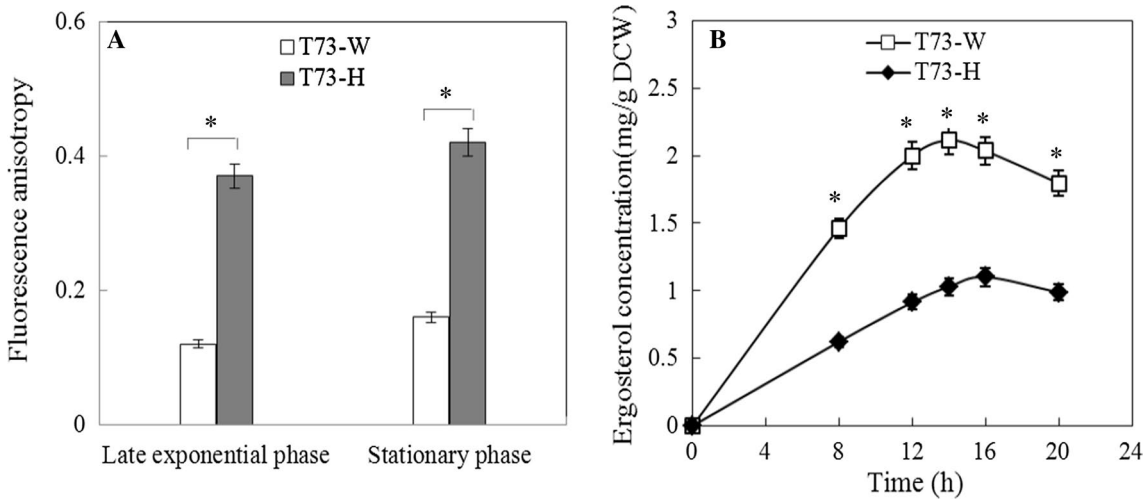

Fig. 3 Fluidity of cell membranes of the parent strain, T73-W, and the recombinant strain, T73-H, at the late-exponential and stationary phases as determined by fluorescence anisotropy of 1,6-diphenyl-1,3,5-hexatriene-stained strains (**a**) and the profiles of the ergosterol concentrations of both strains (**b**). Values are the mean ± standard deviations of three experiments. The *asterisks* indicate a significant difference between the data obtained from strain T73-W and from T73-H ($p < 0.05$, Student's *t* test)

their homeostatic levels (including uptake, distribution and storage) are tightly regulated. Intracellular levels of metal ions are largely determined by the ability of cells to take them up from the environment. A decrease in CM fluidity can reduce this uptake and cause a decrease in the concentration of metal ions in cells [32]. CM fluidity is largely determined by the unsaturated fatty acid and ergosterol content in yeast cells, and lower levels of both functional compounds can cause CM rigidity. In genetically engineered *S. cerevisiae*, carotenoid biosynthesis shares the precursors acetyl-CoA and FPP with unsaturated fatty acids and ergosterol, respectively. Therefore, heterologous carotenoid biosynthesis could decrease the contents of unsaturated fatty acid and ergosterol due to competition for these precursors. This phenomenon has been observed in *Phaffia rhodozyma* [10]. Thus, we suspected that the decrease in metal ions in the recombinant strain was associated with the decrease in CM fluidity. To address this, we first analyzed the membrane fluidities of both strains by measuring fluorescence anisotropy with DPH (Fig. 3a). DPH can anchor in the lipid region of the membrane surface in contact with water and is frequently used to monitor changes in membrane dynamics. Increases in fluorescence anisotropy of 2.08- and 1.63-fold were observed in the recombinant yeast strain compared to that of the parent strain at the late-exponential phase and stationary phases, respectively. These results confirmed that the CM of recombinant yeast was more rigid than that of the parent strain.

Next, we determined the ergosterol (Fig. 3b) and unsaturated fatty acid (Fig. 4) content in both strains. Although the levels of ergosterol in the T73-H strain gradually increased during fermentation, the contents were significantly lower than those of the parent strain. Fatty acid biosynthesis showed a similarly decreasing trend. Total fatty acid contents (C8–C22) in the T73-H strain were 27.0 and 17.9 % lower than in the parent strain at 14 and 16 h of fermentation, respectively. However, the content in the T73-H strain at 20 h was slightly higher than in the parent strain. The major fatty acids in yeast are palmitic acid (16:0), palmitoleic acid (16:1), stearic acid (C18:0) and oleic acid (C18:1). Our results clearly show that the decrease in total fatty acid was primarily caused by the decrease of unsaturated fatty acids (UFAs), which were 57.8, 58.2, and 49.3 % lower in the recombinant strain than in the parent strain at 14 h, 16 h and 20 h, respectively. In comparison, SFA content showed an increased trend with carotenoid accumulation. As a result, the ratio of UFAs/SFAs (unsaturation index), the parameter that determines CM fluidity, was significantly lower in the recombinant strain than in the parent strain (Fig. 4b). These results further support the conclusions presented above. It should be noted that carotenoids themselves might also lead to rigidity of the CM in the recombinant strain because carotenoids can form an integral part of the membrane structure, which can increase the rigidity and mechanical strength of the membrane [33].

Effects of linoleic acid addition on the levels of metal ions and the production of β-carotene

The results obtained here suggest that the reduction of unsaturated fatty acid and ergosterol contents are primarily responsible for the decrease in CM fluidity in the recombinant strain. If the rigidity of the CM leads to a deficiency of metal ions, one would expect that recovery of CM

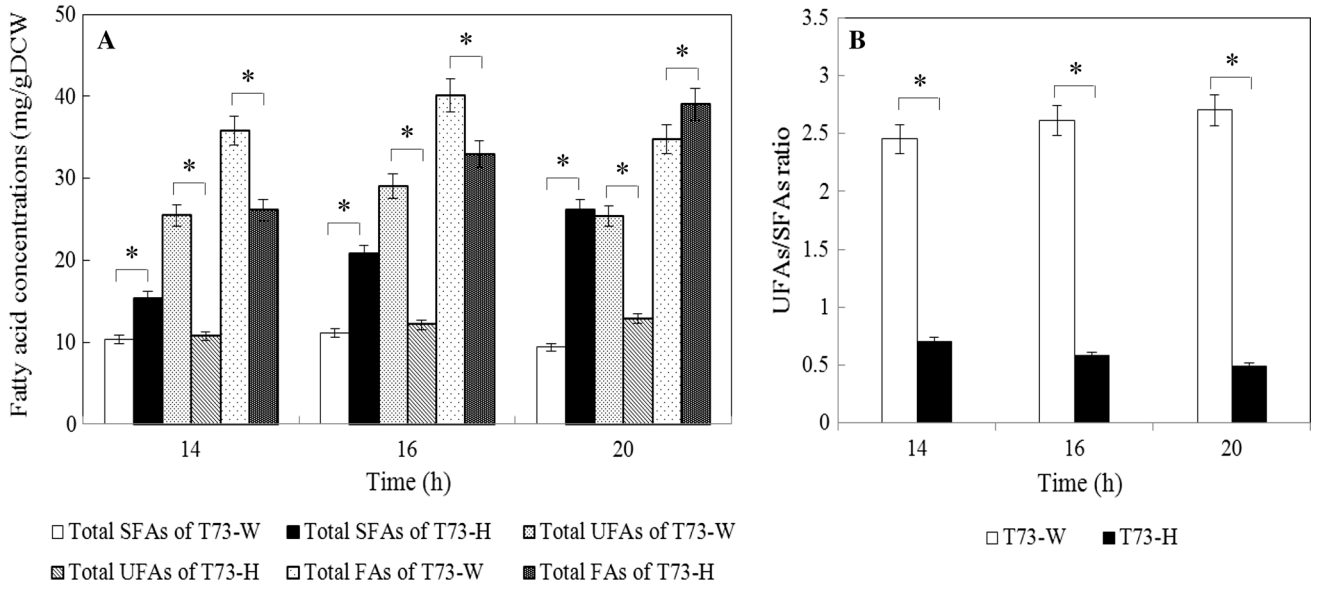

Fig. 4 Fatty acid composition (**a**) and the unsaturation index (**b**) of the parent strain T73-W and the recombinant strain T73-H at different cultivation times. Total fatty acid concentrations were calculated as the sum of C8–C22 (saturated and unsaturated). Total SFAs were calculated as the sum of C16 and C18 and total UFAs were calculated as the sum of C16:1 and C18:1. Values are the mean ± standard deviations of three experiments. The *asterisks* indicate a significant difference between the data obtained from strain T73-W and from T73-H ($p < 0.05$, Student's *t* test)

fluidity could, at least partially, restore metal ion levels in the cells. Previous research has confirmed that the accumulation of linoleic acid (C18:2) in yeast cells by the overexpression of Δ12 fatty acid desaturase can increase CM fluidity [34], while the addition of linoleic acid (C18:2) can lead to the large-scale incorporation of this polyunsaturated fatty acid (to 60 % of total fatty acids) into yeast membrane lipids and increase the fatty acid unsaturation index [32]. We therefore added 0.5 mM linoleic acid (C18:2) to the medium of the T73-H strain to determine if it would improve CM fluidity. The fluorescence anisotropy of treated cells decreased to 0.12, compared to 0.39 for untreated cells, and confirmed that CM fluidity is restored by the incorporation of UFAs into the CM. As expected, measurements of the metal ion contents in recombinant yeast cells indicated that the metal ion levels were restored along with the increase in CM fluidity (Fig. 5). The concentration of Mg^{2+} increased from 773.1 to 1161.4 μg/OD_{660}, Cu^{2+} increased from 3.0 to 6.3 μg/OD_{660}, and Mn^{2+} increased from 1.85 to 2.64 μg/OD_{660}. The concentration of Fe^{2+} increased from 8.4 to 12.4 μg/OD_{660} and even exceeded the value of the untreated parent strain. There was no clear effect on Ca^{2+} or Zn^{2+} concentrations. The increased concentrations of metal ions were also found in the parent strain when treated with C18:2 compared to those of untreated cells. These results strongly suggest that the intracellular concentrations of metal ions are closely associated with CM fluidity, and confirm that the deficiency of metal ions in the recombinant yeast cells resulted from the decreased CM fluidity.

We also measured the β-carotene production in response to the addition of linoleic acid. β-carotene production was slightly increased by the UFA addition, and the highest β-carotene concentration (4.59 mg/g DCW), achieved at 28 h, was 24.3 % higher than that of untreated cells (Fig. 6). Although this increase was not exceptionally large, these data do imply that UFA addition is beneficial to β-carotene biosynthesis. More experiments, such as optimizing the concentration and time of UFA addition, are needed to further highlight the beneficial effect of UFAs on carotenoid biosynthesis. These experiments are in progress in our lab. There have been a few reports on the beneficial effects of fatty acid addition on microbial carotenoid production, but these improvements were usually ascribed to in situ extraction [11, 35, 36]. Recently, Sonntag et al. [36] found that the addition of linoleic acid to the dodecane two-liquid-phase medium is more efficient in increasing the amount of β-carotene extraction from genetically engineered *S. cerevisiae* strains than the addition of dodecane alone [36]. This might be due to the positive effect of linoleic acid on CM fluidity, which favors carotenoid biosynthesis and excretion into the dodecane phase. Normal CM fluidity is essential for normal processes of cellular metabolism, such as facilitating the absorption of essential substances (e.g., oxygen and amino acids) and resisting harsh environmental conditions [21]. Therefore, maintaining normal CM fluidity

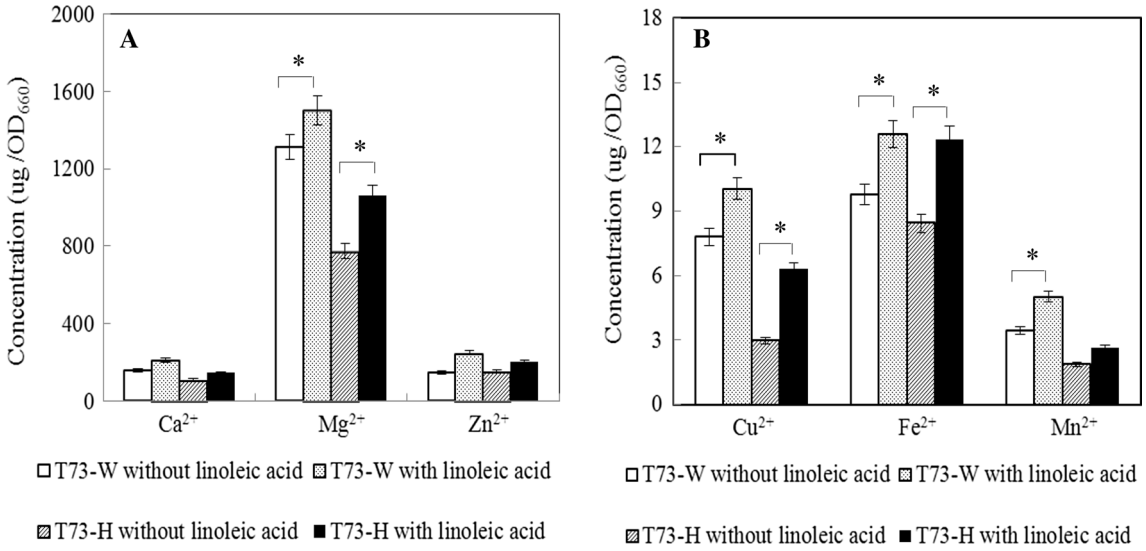

Fig. 5 The concentrations of Ca^{2+}, Mg^{2+}, Zn^{2+} (**a**) and Cu^{2+}, Fe^{2+}, Mn^{2+} (**b**) in the parent strain, T73-W, and the recombinant strain, T73-H, with and without added linoleic acid. Values are the mean ± standard deviations of three experiments. The *asterisks* indicate significant difference between data ($p < 0.05$, Student's *t* tests)

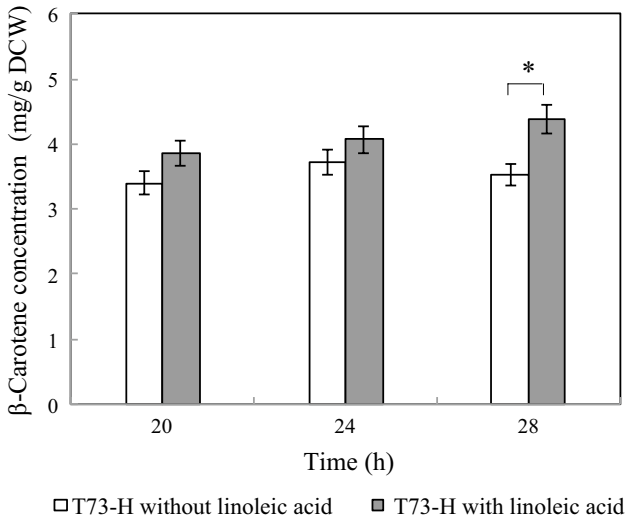

Fig. 6 Effects of adding 0.5 mM linoleic acid on the β-carotene production of the recombinant strain, T73-H. Values are the mean ± standard deviations of three experiments. The *asterisks* indicate significant difference between data ($p < 0.05$, Student's *t* tests)

is important for bio-compound synthesis. This is consistent with the viewpoint that directing FPP towards the isoprenoid and sterol pathways, as a way to maintain the required levels of ergosterol, is essential for effectively increasing heterologous isoprenoid production [37]. Recently, Royce et al. [38] demonstrated that the alteration of membrane properties is an effective strategy to significantly increase the production of bio-renewable fuels and chemicals [38], which agrees with our present conclusions.

There have been several studies suggesting that metal ions such as Mg^{2+} and Mn^{2+} have a stimulating effect on carotenoid production in wild-type microorganisms. This has been ascribed to the fact that Mg^{2+} and Mn^{2+} are essential for the activity of critical enzymes, such as phosphomevalonate kinase (PMK), in the mevalonate pathway [31, 39]. We conducted a preliminary study on whether adding metal ions promoted carotenoid biosynthesis in genetically engineered *S. cerevisiae*. Different concentrations of metal salts, including $CuCl_2$, $FeCl_2$, $MnSO_4$, $CaCl_2$, $ZnSO_4$, and $MgCl_2$, were added to the initial medium. The results showed that, except for Ca^{2+} and Zn^{2+}, the addition of 50 μM Cu^{2+}, 50 μM Fe^{2+}, 10 mM Mn^{2+}, and 4 mM Mg^{2+} resulted in β-carotene concentrations of 4.43, 4.35, 4.71, and 4.65 mg/g DCW, which were 17.8, 15.7, 25.3, and 23.6 % higher than those of untreated cells, respectively. These preliminary data imply that enrichment of metal ions in cells has a beneficial effect on carotenoid production in genetically engineered *S. cerevisiae*. It should be mentioned that in addition to influencing metal ion uptake, decreased CM fluidity would also negatively affect the absorbance of amino acids. Our transcriptional analysis results seemed to verify this conclusion because the genes involved in the transport of amino acids and amino acid derivatives (*AGP1* and *GAP1*) were induced in the carotenoid-producing stains (Table 1). The induction of *AGP1* was also found by Verwaal et al. [11]. The decrease in amino acids in yeast cells is usually accompanied by an increased expression of amino acid transporter genes [40]. This conclusion needs to be further confirmed by a metabolomic approach. Overall, because of the essential role of CM fluidity in the normal

physiological and metabolic processes of yeast cells, more effort should be made to determine how to maintain normal CM fluidity when manipulating the mevalonate pathway as a way to increase carotenoid production in genetically engineered *S. cerevisiae.*

Conclusions

In the present study, we used DNA microarray technology to reveal that the production of high levels of heterologous carotenoid in *S. cerevisiae* can lead to metal ion deficiencies in these cells and that this can induce the expression of genes involved in the transport of metal ions. The decreased fluidity of the CM caused by the decreased UFA and ergosterol contents was confirmed to be primarily responsible for the deficiency of metal ions, as decreased CM fluidity can increase the diffusion barrier for these ions. Considering the importance of CM fluidity in cellular metabolism and physiology, maintaining normal CM fluidity in recombinant *S. cerevisiae* might be essential for effectively increasing carotenoid production. To solve this problem, increasing the unsaturated fatty acid content in cells by exogenous addition or by overexpressing genes that encode fatty acid desaturase could be a potential method to counteract the CM rigidity induced by carotenoid accumulation.

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