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Enhanced production of β-carotene in recombinant *Saccharomyces cerevisiae* by inverse metabolic engineering with supplementation of unsaturated fatty acids

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ABSTRACT

β-Carotene biosynthesis in recombinant *Saccharomyces cerevisiae* was improved using inverse metabolic engineering. Determination of the transcriptional profiles of mevalonate pathway genes showed that transcription of *HMG1*, *ERG8*, *ERG19* and *ERG20* were induced in recombinant strain compared with the parent strain, whereas the expression of *HMG2*, *ERG12* and *IDI* showed no significant differences. The contents of ergosterol and unsaturated fatty acids (UFAs) were decreased in recombinant yeast, and *ERG9* and *OLE1* involved in their syntheses were upregulated, suggesting that an increased demand exists for ergosterol and UFAs in recombinant yeast. Single overexpression of *ERG19*, *ERG12* and *ERG20* led to 11.0–32.2% increments in β-carotene content, but caused 22.9% and 13.6% decrements in overexpression of *ERG8* and *IDI1*, respectively. In comparison, supplementation of 60 mg/l oleic acid and palmitoleic acid led to 83.7% and 130.2% increments of β-carotene content, respectively, in parallel with recovery of UFA contents in cells. These results suggested that single overexpression of most genes in mevalonate pathway is not an effective approach for enhanced carotenoid production. However, intracellular UFA contents are important for carotenoid biosynthesis, and enriching UFA contents by exogenous supplementation or strengthening their biosynthesis might be a promising strategy to improve carotenoid production in recombinant *S. cerevisiae*.

1. Introduction

Carotenoids are a class of pigments of commercial interest that exert important biological functions. In humans, β-carotene is the precursor for vitamin A that might function as an anti-oxidant, has protective properties against cancer, and stimulates the immune system [1]. The use of chemically synthesized β-carotene compounds as food additives or functional cosmetic agents has been severely regulated in recent years, which has increased interest in natural β-carotene and in large-scale fermentative production by *Sphingomonas* sp. (bacteria), *Blakeslea trispora* (fungi) and *Rhodotorula* spp. (yeast) [2–4]. An alternative way of β-carotene production involves the heterologous expression of β-carotene biosynthetic genes in noncarotenogenic microorganisms such as *Escherichia coli, Saccharomyces cerevisiae* and *Candida utilis* [5–7]. In contrast to other hosts, *S. cerevisiae* exhibits an efficient isoprenoid metabolism and is capable of accumulating large quantities of ergosterol in their membranes [8]. Furthermore, the *S. cerevisiae* strain is categorized as a GRAS organism, which is desirable for the production of β-carotene for pharmaceutical, nutritional and feed applications [6].

β-Carotene is synthesized through the mevalonate (MVA) pathway in *S. cerevisiae* (Fig. 1). In the initial step, three molecules of acetyl-CoA (which is also a substrate of fatty acid synthesis) undergo condensation to yield acetoacetyl-CoA, which is subsequently converted into 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) by the gene product of 3-hydroxy-3-methylglutaryl coenzyme A synthase (HMGS). Mevalonate is generated and catalyzed by the action of the rate-limiting enzyme HMG-CoA reductase (HMGR). Consecutive phosphorylations and decarboxylation of mevalonate result in isopentenyl pyrophosphate (IPP, C5) and its isomer dimethylallyl pyrophosphate (DMAPP, C5). IPP and DMAPP are further condensed by prenyl transferases to synthesize geranyl pyrophosphate (GPP, C10), farnesyl pyrophosphate (FPP, C15) and geranylgeranyl pyrophosphate (GGPP, C20)

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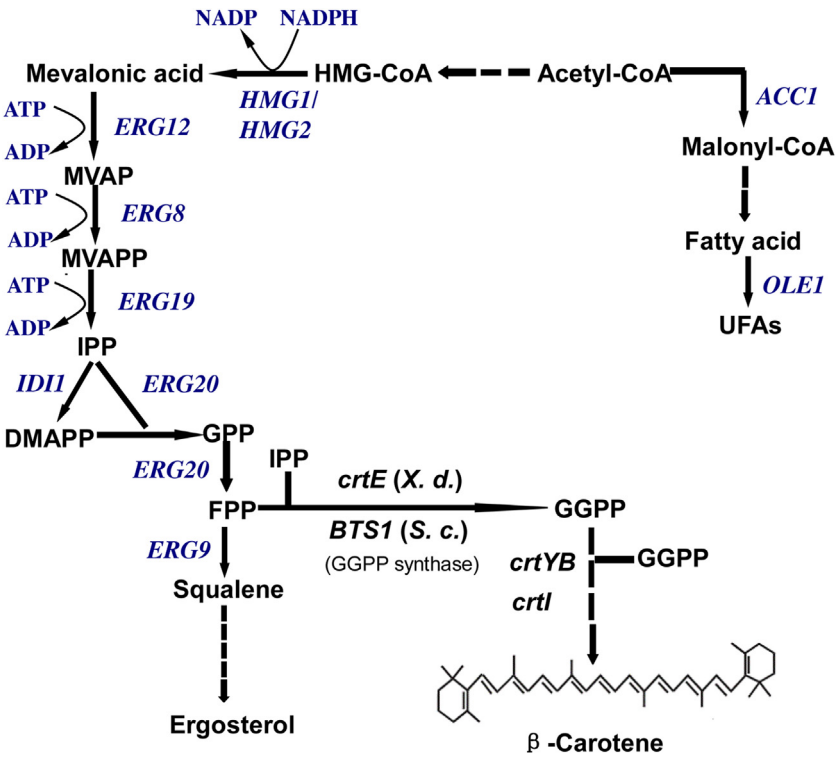

Fig. 1. Biosynthetic pathway of β-carotene in recombinant *S. cerevisiae*. The dashed lines indicate multiple step reactions. *HMG1* and *HMG2*: HMG-CoA synthase, *ERG12*: mevalonate kinase, *ERG8*: phosphomevalonate kinase, *ERG19*: diphosphomevalonate decarboxylase, *IDI1*: IPP:DMAPP isomerase, and *ERG20*: FPP synthase. Fatty acids pathway: *ACC1*: acetyl CoA carboxylase; *OLE1*: stearoyl-CoA desaturase. Carotenoids pathway: *CrtE*: GGPP synthase, *BTS1*: *S. cerevisiae* GGPP synthase, *CrtYB*: encoding bifunctional phytoene synthase and lycopene cyclase, *CrtI*: Phytoene desaturase. MVAP: phosphomevalonate; MVAPP: diphosphomevalonate; IPP: isopentenyl pyrophosphate; DMAPP: dimethylallyl pyrophosphate; GPP: geranyl pyrophosphate; FPP: farnesyl pyrophosphate; GGPP: geranylgeranyl pyrophosphate. UFA: unsaturated fatty acid.

[9]. Co-expression of the three exogenous genes GGPP synthase (*crtE*), bifunctional phytoene synthase and lycopene cyclase (*crtYB*) and phytoene desaturase (*crtI*) from *Xanthophyllomyces dendrorhous* drive two GGPP molecules into the carotenoids pathway and synthesize orange-colored β-carotene through condensation, dehydrogenation and two cyclization reactions [6].

A sufficient supply of precursors in the mevalonate pathway is crucial for accumulation of carotenoids. Therefore, manipulations of the central metabolic pathway for sufficient supply of precursors and cofactor are essential for enhancement of carotenoid production, e.g., overexpression of the key enzyme *HMG1* [6,10], increase cofactor (ATP and NADPH) supplies [5] and downregulation of *ERG9*, which limits ergosterol accumulation and drives additional FPP into carotenoid biosynthesis [7]. It is well known that the mevalonate pathway provides many functional compounds, including ergosterol, dolichols and ubiquinones, and thus, it is subject to tight feedback control at multiple levels (gene transcription, mRNA translation, enzyme activity and protein stability) [9,11]. In addition to *HMG1*, it has been reported that overexpression of *ERG20* can promote heterologous sesquiterpene production [12]. However, information on manipulation of other mevalonate pathway genes in carotenoid biosynthesis by *S. cerevisiae*, such as *ERG8*, *ERG12*, *ERG19* and *IDI1*, is still lacking.

Revealing the transcriptional regulation of the mevalonate pathway and metabolite changes of *S. cerevisiae* in response to carotenoid biosynthesis can aid in identifying the potential factors that limit carotenoid formation and designing a more effective strategy for improved carotenoid biosynthesis [13]. To achieve this goal, inverse metabolic engineering is a useful technique that can identify the cause of a desired phenotype and has been successfully applied in different microbial cell factories to improve production of bio-compounds [14,15]. In this study, based on the theory of inverse metabolic engineering, the transcriptional profiles of mevalonate pathway genes and intermediate products (ergosterol and fatty acids) were compared for a recombinant *S. cerevisiae* strain that produces β-carotene and its parent strain. Based on the obtained results, the effects of overexpression of single genes in the mevalonate pathway and exogenous addition of UFA on cell growth and β-carotene production were investigated.

2. Materials and methods

2.1. Yeast strains and medium

The wild-type parent strain FY1679-01B (*MAT*a; *ura3-52*) and FY1679-03D (*MAT*a; *ura3*-52; *leu2*Δ 1) isogenic to the S288C strain [16] were obtained from EUROSCARF (Frankfurt, Germany) and were used to construct the β-carotene producing strains FY-14-05 and FY20-01, respectively. Minimal synthetic defined medium was used for selection of yeast transformants [17]. *E. coli* DH5a (Invitrogen, Carlsbad, CA) were used as the host for the propagation and manipulation of plasmids.

2.2. Plasmid and yeast transformation

The strains and plasmids used in this study are listed together with their sources and relevant genotypes in Table 1. The integration vectors YIplac211YB/I/E* carrying the carotenoid biosynthesis genes were kindly provided by Verwaal et al. [6] and include the genes *crtYB* (encodes a bifunctional phytoene synthase and lycopene cyclase), *crtI* (phytoene desaturase) and *crtE* (GGPP synthase) cloned from *X. dendrorhous*. The expression levels of the three genes were driven by the *S. cerevisiae* GPD strong constitutive promoter and CYC1 terminator. For yeast transformation,