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A genetic screen for increasing metabolic flux in the isoprenoid pathway of *Saccharomyces cerevisiae*: Isolation of *SPT15* mutants using the screen

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ABSTRACT

A genetic screen to identify mutants that can increase flux in the isoprenoid pathway of yeast has been lacking. We describe a carotenoid-based visual screen built with the core carotenogenic enzymes from the red yeast *Rhodosporidium toruloides.* Enzymes from this yeast displayed the required, higher capacity in the carotenoid pathway. The development also included the identification of the metabolic bottlenecks, primarily phytoene dehydrogenase, that was subjected to a directed evolution strategy to yield more active mutants. To further limit phytoene pools, a less efficient version of GGPP synthase was employed. The screen was validated with a known flux increasing gene, *tHMG1*. New mutants in the TATA binding protein *SPT15* were isolated using this screen that increased the yield of carotenoids, and an alternate isoprenoid, α-Farnesene confirming increase in overall flux. The findings indicate the presence of previously unknown links to the isoprenoid pathway that can be uncovered using this screen.

1. Introduction

Isoprenoids or terpenoids represent the largest class of natural products with more than 40,000 known structures (Bohlmann and Keeling, 2008). Many of these terpenoids are of immense commercial value. Their biosynthesis in heterologous hosts offers an alternative to the chemical synthesis or the extraction from their natural sources (Farhi et al., 2011; Herrero et al., 2008; Keasling, 2010). *Saccharomyces cerevisiae* is one of the choice organisms as heterologous host for terpenoids (Hong and Nielsen, 2012; Nevoigt, 2008).To increase the yield of isoprenoids in yeast, previous efforts have focused on manipulating the mevalonate pathway. Using known information about the mevalonate-isoprenoid pathway, three potential targets were identified as being potential bottlenecks for isoprenoid biosynthesis, HMG-CoA reductase (*HMG1*), the transcription factor *UPC2* and the ergosterol branch point *ERG9*. Using truncated *tHMG1* (that lacks feedback regulation) (Asadollahi et al., 2010; Gardner and Hampton, 1999; Ro et al., 2006; Westfall et al., 2012; Zhou et al., 2012), a hyperactive transcription factor *upc2-1* (that increases expression of the mevalonate pathway) (Ro et al., 2006; Westfall et al., 2012), or reduced expression of *ERG9* (that prevents isoprenoids from branching off) (Asadollahi et al., 2010; Babiskin and Smolke, 2011; Paradise et al., 2008; Ro et al., 2006; Westfall et al., 2012), increased flux has been demonstrated and the yield of isoprenoids further increases when these different mutations are combined. However, in the cell, metabolic pathways are interconnected and tightly regulated (Szappanos et al., 2011), and it is possible that besides the mevalonate pathway genes, there may be other genes which affect directly or indirectly the yield of carotenoids or other terpenoids produced in yeasts. To identify these, a good genetic screening method is required. As carotenoids are coloured compounds, their production by yeast cells provides a good visual phenotype, and this has been extensively exploited in the past (Mitchell et al., 2015; Schmidt-Dannert et al., 2000; Wang et al., 2009; Xie et al., 2014). However, surprisingly, despite their extensive use in a variety of different screens and assays, their development as a measure of isoprenoid flux has remained unsuccessful so far.

A few groups have attempted to increase the metabolic flux in the isoprenoid pathway using this carotenoid based visual screen using the carotenogenic enzymes from *Xanthophyllomyces dendrorhous* (Ozaydin et al., 2013; Verwaal et al., 2007; Yuan and Ching, 2014). However these studies have met with limited success. It was observed that upon increasing the flux in this pathway through known flux increasers such as *tHMG1*, a decrease (rather than an increase) in pigmentation was observed (Verwaal et al.,

Abbreviations: T, TEF promoter; C, CYC promoter; GGPPS, Geranylgeranyl diphosphate synthase; PSY1, phytoene synthase; CRTI, phytoene dehydrogenase

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2007; Yuan and Ching, 2014). Estimation of carotenoids revealed that the decrease was most likely due to accumulation of the colourless intermediate, phytoene which masked any increase in colour due to higher β-carotene (Verwaal et al. 2007). A visual carotenoid based screen has also been employed to screen the yeast deletion collection to identify gene deletions that could improve isoprenoid production (Ozaydin et al. 2013). Although the study succeeded in obtaining deletion mutants with more β-carotene, it did not appear to be a validated screen for isoprenoids since the higher pigmentation yielding deletion mutants did not yield increased levels of an alternate isoprenoid, bisabolene.

The red yeasts belonging to the *Rhodotorula* spp., *Rhodosporidium* spp. and *Sporobolomyces* spp. have an intense red colour and are considered to be the yeasts with the highest β-carotene levels (Mata-Gomez et al., 2014). In addition to β-carotene, these yeasts produce the carotenoids- torulene and torularhodin. Owing to the high production of carotenoids from these yeasts, the possibility that the enzymes from these organisms might have evolved to be more efficient seems a likely possibility. In attempting to develop a genetic screen for isoprenoid/carotenoid production in *Saccharomyces cerevisiae*, we have sought to use enzymes from these yeasts in place of *X. dendrorhous.* Based on the recently released genome sequences of *Rhodosporidium toruloides* by multiple groups (Kumar et al., 2012; Zhu et al., 2012) we identified and carried out codon-optimised expression of the genes for the core biosynthetic carotenogenic enzymes upto β-carotene from *R. toruloides* into *S. cerevisiae*. Although the core carotenogenic enzymes of *R. toruloides* were more efficient, they still lacked sufficient capacity of pulling increased flux in the pathway through it, and a metabolic bottleneck at phytoene dehydrogenase, *Rt*CRTI, was identified as the rate limitng step. *Rt*CRTI was subjected to a directed evolution strategy and from a mutant library variant enzymes with enhanced activity were isolated. As phytoene levels needed to be further decreased, we used a less efficient version of the precursor enzyme GGPP synthase on a weaker promoter to relieve phytoene buildup to eventually yield a combination that could function as a genetic screen, as validated by over expression of *tHMG1* in this background. The developed screen enabled the identification of mutants of TATA binding protein *SPT15*, that increased yields of β-carotene. The isolated *spt15* mutants could also enhance the levels of an alternate isoprenoid, the sesquiterpene α-Farnesene suggesting that the mutants were in fact enhancing isoprenoid flux and were not exclusive to carotenoids. These results, which describe and validate a carotenoid-based screen for isoprenoid flux in yeast, are described in this report.

2. Materials and methods

2.1. Plasmid vectors, cloning of genes and transformation

The yeast centromeric plasmids p416TEF, pRS313TEF, pRS314TEF, pRS315TEF as well as the same series with the CYC promoter were used for cloning and expression of carotenogenic genes. pRS313TEF, pRS314TEF and pRS315TEF were constructed by excising the MCS and TEF promoter regions from p416TEF plasmid and cloning into pRS313, pRS314 and pRS315 respectively. The genes for Geranylgeranyl diphosphate (GGPP) synthase (*Rt*GGPPS), Phytoene synthase (*Rt*PSY1) and Phytoene dehydrogenase (*Rt*CRTI) of *R. toruloides* were codon optimised by using EnCor Biotechnology Inc. (http://www.encorbio.com/protocols/Codon.htm) software and custom synthesised by GenScript USA. These genes are cloned in pRS315TEF, p416TEF and pRS314TEF respectively. *Rt*GGPPS was cloned at the *Xba*I and *Bam*HI sites of pRS315TEF to construct pRS315TEF- *Rt*GGPPS, *Rt*PSY1 was cloned at *Bam*HI and *Xho*I sites of p416TEF to yield p416TEF- *Rt*PSY1 while *Rt*CRTI is cloned at *Bam*HI and *Sal*I site to construct pRS314TEF-*Rt*CRTI. For over expression of truncated HMG CoA reductase 1 (*tHMG1*), the C-terminal catalytic region (1575 bp) was amplified from *S. cerevisiae* genomic DNA using *tHMG1*-FP and *tHMG1*-RP and the amplified PCR product was cloned at the *Bam*HI and *Xma*I sites of pRS313TEF to construct pRS313TEF-*tHMG1*. For construction of p416CYC- *Rt*PSY1, p416TEF-*Rt*PSY1 is digested with *Sac*I and *Bam*HI to excise the TEF promoter and ligated with *Sac*I and *Bam*HI digested CYC1 promoter from p414CYC1 vector. pRS314CYC-*Rt*CRTI was constructed similarly. pRS315CYC- *Rt*GGPPS was constructed from pRS315TEF-*Rt*GGPPS by digesting with *Xba*I and *Sac*I to excise the TEF promoter and ligated with *Xba*I and *Sac*I digested CYC1 promoter of p414CYC1 vector. *SPT15* was amplified from *S. cerevisiae* ABC 276 strain by using the forward and reverse primers and cloned in the *Bam*HI and *Xho*I sites of pRS313TEF. The cDNA for α-Farnesene synthase (Locus AT4G16740 and clone no. U88221) from *Arabidopsis thaliana* was obtained from TAIR database, USA. It was PCR amplified and subcloned in *Xba*I and *Bam*HI site of pRS315TEF to make the construct pRS315TEF-*At*FS. All these constructs were transformed into *S. cerevisiae* strain (ABC276) by Lithium acetate method (Sambrook, 1989). All the primers and plasmids constructed in this study are indicated in Table S1 and S2.

2.2. Strains and media

Escherichia coli strain DH5α was used as cloning host. *S. cerevisiae* strains CEN. PK2-1C (Euroscarf accession no. 30000 A) -*MAT a, ura 3-52,trp 1-289, leu2-3_112, his3Δ1, MAL 2-8^c*, SUC2 and ABC 276 which is a derivative of S288c strain with genotype *MAT α ura 3-52 leu2Δ1 his3Δ200 trp1 lys2-801* are used in this study. The strain was derived from tetrad analysis of diploids made between BJ5418 and BJ5458 strains which are obtained from the Beth Joan laboratory. These strains were maintained on yeast extract, peptone and dextrose (YPD) media. For culturing yeast-synthetic defined media (SD) containing yeast nitrogen base (YNB) without ammonium sulphate 0.15% (w/v) and amino acids supplemented with appropriate amino acids and 0.5% (w/v) ammonium sulphate and 2% (w/v) D-glucose was used.

2.3. Extraction of carotenoids and analysis by HPLC

Extraction of carotenoids were carried out as described earlier (Moline et al., 2012) with some modifications. Essentially, yeast cells were grown in 100 mL SD media supplemented with appropriate amino acids and grown at 30 °C with shaking (250 rpm). After five days, cells were harvested and washed with deionized water and kept at −20 °C. To the frozen pellet was added 3 mL of Dimethyl sulphoxide (DMSO), vortexed for 1 min and incubated at 55 °C in the water bath for 1 h. 1 g 0.50–0.75 mm glass beads were added, and cells were broken using glass bead beater. Cells were centrifuged to remove the cell debris. Acetone was added to the pellet, vortexed and centrifuged and the process repeated till the pellet becomes colourless. The acetone and DMSO fractions were mixed with an equal amount of Hexane. The coloured hexane layer was collected after separation of two layers. The hexane layer was washed with distilled water and then with brine solution twice. The coloured hexane layer was collected. The solvent was evaporated under rotary evaporator to dryness in dim light and was dissolved in 1 mL hexane for analysis by high performance liquid chromatography (HPLC). HPLC separation and quantification was performed on Waters System using C_{18} – 5 μm intersil ODS-P, 250 × 4.6 mm column (LCGC) using solvent acetonitrile:methanol:2-propanol (85:10:5 v/v) with flow rate 1 mL/min at 32 °C. Separated carotenoids were detected by photodiode array detector. Quantification of carotenoids was done using a standard curve prepared for β-carotene, lycopene and phytoene. Standards