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Construction of a Controllable β-carotene Biosynthetic Pathway by Decentralized Assembly Strategy in *Saccharomyces cerevisiae*[†]

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Abstract

Saccharomyces cerevisiae is an important platform organism for the synthesis of a great number of natural products. However, the assembly of controllable and genetically stable heterogeneous biosynthetic pathways in *S. cerevisiae* still remains a significant challenge. Here, we present a strategy for reconstructing controllable multi-gene pathways by employing the *GAL* regulatory system. A set of marker recyclable integrative plasmids (pMRI) was designed for decentralized assembly of pathways. As proof-of-principle, a controllable β-carotene biosynthesis pathway (~16 kb) was reconstructed and optimized by repeatedly using *GAL10-GAL1* bidirectional promoters with high efficiency (80%-100%). By controlling the switch time of the pathway, production of 11 mg/g DCW of total carotenoids (72.57mg/L) and 7.41mg/g DCW of β-carotene was achieved in shake-flask culture. In addition, the engineered yeast strain exhibited high genetic stability after 20 generations of subculture. The results demonstrated a controllable and genetically stable biosynthetic pathway capable of increasing the yield of target products. Furthermore, the strategy presented in this study could be extended to construct other pathways in *S. cerevisisae*.

Keywords: β-carotene; decentralized assembly; pMRI; biosynthetic pathway; functional module

Introduction

Saccharomyces cerevisiae is an attractive microbial cell factory due to its inherent safety, industrial robustness and ease of genetic manipulation (Siddiqui et al. 2011). Because of this, many have sought to produce high value-added products such as biofuels and pharmaceuticals from yeast by introducing multi-gene biosynthetic pathways from heterologous organisms (Szczebara et al. 2003; Zhou et al. 2012). However, efficiently rebuilding controllable and genetically stable heterologous pathways in yeast was still challenging in metabolic engineering (Blount et al. 2012).

Typically, overexpression of targeted genes is the principal strategy for enhancing the metabolic flux and the yield of products, so constitutive strong promoters are often selected for gene expression (Partow et al. 2010; Sun et al. 2012; Verwaal et al. 2007). Constitutive overexpression can offer a high yield of protein but at the cost of increased metabolic burdens on the host cell. In addition, if the chemical or intermediates of a foreign biosynthesis pathway is toxic to the host cell, constitutive overexpression can result in decreased production of the desired compound (Keasling 2012; Nevoigt 2008; Pitera et al. 2007). Therefore, finding more flexible approaches for the controlled expression of targeted genes as well as optimizing the switch time of the pathway has garnered increasing interest in the area of metabolic engineering (Krivoruchko et al. 2011; Nevoigt 2008).

Unlike *Escherichia coli*, in which coexpression of several genes can be controlled by an operon, the expression of each gene in yeast requires an individual promoter and terminator. Although a few synthetic regulatory networks have been developed (Blount et al. 2012; Seo et al. 2013), the regulation of multiple genes in a pathway is still hard to mediate. Consequently, employing the native regulation system in yeast to control the expression of multiple genes is a more attractive

alternative (Da Silva and Srikrishnan 2012; Krivoruchko et al. 2011; Nevoigt 2008).

The galactose regulatory network in *S. cerevisiae* is one of the most well-characterized transcriptional induction systems whose induction and repression are tightly regulated by galactose and glucose (Johnston et al. 1994; Lohr et al. 1995). Since the promoters of *GAL1* and *GAL10* in this system can be induced at least 1000-fold (Giniger and Ptashne 1988), high-copy 2μ plasmids carrying these two promoters has previously been applied for the recombinant protein production (Choi et al. 1994; Romanos et al. 1992) and biosynthetic pathway construction in *S. cerevisiae* (Maury et al. 2008; Shiba et al. 2007; Steen et al. 2008). Nevertheless, using plasmids to construct a biosynthetic pathway is hindered by drawbacks such as "plasmid instability" , "plasmid burden" (Karim et al. 2012; Zhang et al. 1996) and special medium requirements. Therefore, genomic integration is often preferred.

In this study, we sought to employ the galactose regulatory network for constructing a controllable and genetically stable pathway in yeast. All of the genes in the pathway were integrated into the genome and their expression was controlled by repeatedly using the *GAL1-GAL10* bidirectional promoters. However, repeated sequences gathered at a single site are prone to recombination and can result in instability of the integrated structure (Blount et al. 2012; Kolodner et al. 2002). To circumvent this problem, we integrated different sections of the pathway into separated genomic loci. This type of integration strategy is denoted as "decentralized assembly" in this work. A set of ready-to-use marker recyclable integrative plasmids (pMRI) was also designed to support the decentralized assembly strategy. As proof of principle, an optimized β-carotene biosynthetic pathway involving six open reading frames (ORF) was constructed under the control of GAL promoters in *S. cerevisiae*. To improve the production of carotenoids, the switch time of the pathway was adjusted by controlling the glucose supply in media. In addition, the

efficiency of the decentralized assembly strategy and the stability of the engineered yeast strain were also investigated.

Materials and Methods

Strains, Media, Reagents and Transformation Methods

S. cerevisiae strain BY4741 (Brachmann et al. 1998) was used as a host for β-carotene biosynthetic pathway introduction and genome DNA preparation. Genes for the β-carotene pathway construction were amplified from the cDNA of *Xanthophyllomyces dendrorhous* (As2.1557). YPD medium (1% yeast extract, 2% Peptone and 2% D-glucose), YPG medium (1% yeast extract, 2% Peptone and 2% D-galactose) and YPDG medium (1% yeast extract, 2% Peptone, D-glucose and Glycerol) were used to cultivate yeast. YPD medium containing 200 μg/ml geneticin (G418) was used for selection of the *KanMX* marker. The yeast cells carrying plasmids with the URA selectable marker were selected and cultivated by synthetic complete drop-out medium with 2% D-glucose or galactose without uracil (SD-URA and SG-URA, respectively). Plasmids or gel-purified DNA fragments were transformed into *S. cerevisiae* by electroporation at 1.5 kV in a 0.2 cm gap electroporation cuvette by using an Eppendorf Eporator 2510. Plasmids were transformed into *E. coil* DH5α by the heat shock method (Chung et al. 1989). All restriction enzymes, T4 DNA ligase and primers were purchased from Sangon Biotech (Shanghai, China). PrimeSTAR HS DNA polymerase was purchased from Takara (Dalian, China). The standard β-carotene was purchased from Sigma (Sigma Aldrich, USA).

Construction of the pMRI Plasmids

We amplified the necessary backbone components from PUG6 (Guldener et al. 1996), p416GPD (Mumberg et al. 1995), and PESC-URA (Stratagene) for basic pMRI plasmid construction. A

combination of standard subcloning techniques and the circular polymerase extension cloning (CPEC) method (Quan and Tian 2011) were adopted for plasmid construction. Primers used for pMRI plasmid construction and corresponding amplicons are listed in SI-1 (supporting information). To obtain the pMRI plasmids for decentralized assembly, the basic vectors pMRI-01 and pMRI-02 were constructed by using *loxP-KanMX*, *pBR322ori-Amp*^{r} and *loxP* as basic elements. The detailed construction procedures and sequence information for two plasmids are provided in supporting information SI-2 and SI-10. The pMRI plasmids (Fig. 1) for gene integration were generated using the method described in supporting information SI-3.

Construction of Functional Modules

The *ctrE* (GGPP synthase), *crtYB* (encodes a bifunctional phytoene synthase and lycopene cyclase), *CrtI* (phytoene desaturase) (Verdoes et al. 1999a; Verdoes et al. 1999b) genes were amplified from the cDNA of *X. dendrorhous* (As2.1557). The 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase was considered as a rate-limiting step in the MVA pathway (Polakowski et al. 1998). Its catalytic domain (*tHMG1* gene) was amplified from the genomic DNA of *S. cerevisiae* BY4741. Genes were cloned into pMRI plasmids pMRI-21, pMRI-31 and pMRI-32 to generate functional modules (Tab. 1). Details of the construction processes are provided in Supporting Information SI-4. The constructed modules were subsequently used to rebuild a controllable β-carotene pathway in yeast.

Construction of Carotenoid Production Yeast Strains

To integrate the *GAL*-regulated system-dependent β-carotene biosynthesis pathway into the yeast genome, we transformed the functional modules in a stepwise manner (Fig. 2). The removal of the *KanMX-pBR322ori* region was carried out by Cre/*loxP* recombination (Gueldener et al. 2002). First, pMRI-31-*crtYB-crtI* was digested with *Sfi*I and integrated into the *HO* locus (SI-5 A) to generate

the strain YXWP-14. The *crtE* and *tHMG1* (Verwaal et al. 2007) were integrated into the *HMG1* locus (SI-5 B) by transforming the *Xba*I linearized pMRI-21-*crtE-tHMG1* to generate the new strain YXWP-40. Because *crtYB* and *crtI* were found to be rate-limiting steps in the β-carotene biosynthesis pathway, another copy number of both *crtYB* and *crtI* were integrated into the *Ty4* loci in YXWP-40 by transforming *Sfi*I linearized pMRI-32-*crtYB-crtI*, and the new strain was designated as YXWP-47.

Typically, inducing gene expression by galactose in yeast can be expensive when scaled up for industrial purposes. In order to address this issue, we constructed a strain in which the switch of the introduced pathway could be controlled by glucose. This was achieved by disrupting the *GAL80* gene in YXWP-47 by using a PCR-mediated method (Wach 1996). The disruption cassette, which consisted of a URA selection marker and the upstream and downstream homologous region of the *GAL80* gene ORF, was constructed by overlapping PCR. Primers for the construction of the disruption cassette are listed in SI-1. The *GAL80* gene in YXWP-47 was deleted by transforming the disruption cassette and selected on a SD (URA^-) plate. The new strain was designated as YXWP-53.

Analysis of *KanMX-pBR322ori* Rescue Efficiency and Strain Genotype

Because the new structure *loxP-KanMX-pBR322ori-loxP* for Cre/*loxP* recombination was designed in pMRI plasmids, the pop-out efficiency of this structure was measured by replica plating on YPD and YPD (+G418) (Guldener et al. 1996). The efficiency of module integration events and the genotype of the strains were analyzed by PCR. For each round of integration and marker recycling, ten colonies were picked randomly and used for PCR analysis. This way, PCR products of the expected size would be obtained only if the integration events and marker rescue were successful. Primers used for genotype verification and the size of the expected PCR products for different

strains are provided in the supporting information SI-6.

Cultivation in Shake-Flasks

Colonies of recombinant yeast strains were picked into 3 ml of YPD liquid medium and the preculture was grown at 230 rpm and 30 °C in a shaking incubator. The shake-flask cultivations were inoculated to an initial OD_{600} of 0.05 from the precultures and then grown for 72 h using the same condition. The strains YXWP-14, YXWP-40, YXWP-47 were cultivated in YPG for carotenoid production. To cultivate YXWP-53, YPD or YPDG containing different concentrations of glucose and glycerol (2% glucose, 1.5% glucose + 0.5% glycerol, 1% glucose + 1% glycerol, 0.5% glucose + 1.5% glycerol, 2% glycerol) were used.

To investigate the fermentation characterization of YXWP-53 in YPD and YPDG broth, the carotenoid accumulation and glucose concentration in different growth stages were measured. The concentration of glucose in medium was measured by an enzyme-coupled glucose assay kit (Rsbio, China). A spectrophotometer (UNIC, China) was used to monitor cell growth by measuring the optical density at 600 nm.

Analysis and Quantification of Carotenoids

The content of the carotenoids was expressed as milligrams per gram of dry cell weight (mg/g DCW) and milligrams per liter of fermentation broth (mg/l). An aliquot of culture was centrifuged for 5 min at 4000 g, and then washed by water. The washed cells were harvested and dried at 80 °C to a constant weight for measuring the dry cell weight. Another aliquot of culture was used to extract carotenoids. Cell precipitates were resuspended in 1 ml of 3 N HCl, heated in a boiled water bath for 3 min and then cooled in an ice-bath for 3 min. The cracked cells were washed once with water and harvested by centrifugation. After removal of the supernatant, the cells were resuspended in 4 ml acetone and vortexed for 5 min. The acetone extracts were centrifuged and filtered with a

4.5 μm-pore-size filter for subsequent analysis.

The content of total carotenoids was measured by a spectrophotometer at a wavelength of 450 nm and calculated by using an extinction coefficient of 2500 ($A^{1\%}$=2500) (Scott 2001). The analysis of β-carotene was performed by HPLC (SHIMADZU LC-20 AT) equipped with Kromasil C18 column (4.6 mm × 250 mm) and UV/VIS detection at 450 nm. The mobile phase consisted of acetonitrile-methanol-isopropanol (50:30:20 v/v) with a flow rate of 1 mL/min at 40 °C.

Stability Analysis of the Assembled Pathway

To evaluate the stability of the constructed strain, YXWP-53 was chosen for subculture. Three colonies were separately picked into individual tubes containing 5 ml of YPD medium. Subsequently, twenty generations of subculture were performed. The cultures of the first, fifth, tenth and twentieth generations were used as seed for shake-flask cultivation, respectively, and spread on YPD plates to evaluate the ratio of colonies with the capability to produce carotenoids.

Results

Design of the Controllable Biosynthetic Pathway

S. cerevisiae can utilize available nutrients in the medium by inducing or repressing relative signaling pathways (Zaman et al. 2008). In this investigation, two main signaling pathways, the Snf1 network, which is responsible for glucose repression (Christensen et al. 2009; Zaman et al. 2008), and the Gal4p–Gal80p regulatory axis, which is responsible for galactose utilization (Johnston et al. 1994; Traven et al. 2006), were employed for constructing a controllable biosynthetic pathway. All genes integrated in the pathway were under the control of *GAL1-GAL10* bidirectional promoters; therefore, their transcription could be regulated by Gal4p (Giniger and Ptashne 1988). Since the carbon source in the medium and the binding of Gal80p were the two

main factors affecting the activation of Gal4p, the switch time of the assembled pathway in a *GAL80* deleted strain could be controlled via the supplementation of a carbon source. Fig. 3 describes the controllable β-carotene biosynthetic pathway in an engineered yeast cell.

Furthermore, promoters were used repeatedly, and two copies of the gnens *crtYB* and *crtI* were integrated in the designed pathway. To avoid undesirable recombination and gene deletion caused by these repeated regions, we employed the decentralized assembly strategy to rebuild the pathway. In this study, the pMRI-21, pMRI-31 and pMRI-32 plasmids (Fig. 1) were constructed and used for controllable pathway assembly. Unlike the traditional pRS series integrating vector (Brachmann et al. 1998), whose selectable marker and replication origin are retained in the genome after integration, the *loxp-KanMX-pBR322ori-loxp* structure in the pMRI plasmids could be readily removed after each round of integration. This guaranteed the recycling of the *KanMX-pBR322ori* region and minimized the unnecessary contamination of bacterial DNA in the yeast genome.

The Efficiency of the Decentralized Assembly

It is generally believed that if same sequences are used to integrate repeatedly, the previously integrated parts in the genome may interfere in subsequent rounds of integration and can result in incorrect recombination (Johansson and Hahn-Hagerdal 2002). We therefore analyzed the efficiency of module integration events during the process of strain construction. Generally, 50-200 colonies appeared on an agar plate after 2-3 days of incubation at 30 °C. The ratio of the right integration events was about 80%-100% in each round of assembly, even though they shared the same T_{ADH1}-MCS1-P_{GAL10}-P_{GAL1}-MCS2-T_{CYC1} plasmid backbone and *crtYB-crtI* construction. These results suggested that the long homologous arms designed in the pMRI plasmids could guarantee a higher accuracy for directed integration.

The marker recycling efficiency of pMRI plasmids was also tested. Typically, 80-90% of

colonies were popped-out successfully after growth for 2 h in galactose-containing medium (SI-7. A). However, *loxP* sites were added into the genome during each round of integration since the Cre/*loxP* system was used in the decentralized assembly strategy, which posed the risk of chromosomal rearrangement when carrying out the marker recycling process (Delneri et al. 2000). For this reason, we investigated the probability of this risk when recycling the marker in YXWP-47. To our surprise, no chromosomal rearrangement event was detected in ten analyzed clones, even though four *loxP* sites coexisted during the marker recycling process (SI-7. B). This result indicated that the risk of chromosomal rearrangement was small，and that many rounds of integration could be carried out if a longer pathway needed to be constructed using the decentralized assembly strategy.

Analysis of the Assembled Pathways in Engineered Yeast

Because different modules were integrated into the yeast genome, the strains BY4741, YXWP-14, YXWP-40, YXWP-47 presented different colors when growing on the YPG plate (Fig. 4). The result of spectrophotometric measurements confirmed that 30 μg/g DCW of total carotenoids was accumulated when only *crtYB* and *crtI* were expressed in YXWP-14 (Tab. 2). Overexpression of *tHMG1* and *crtE* lead to a significant improvement of carotenoid production (959 μg/g DCW) in YXWP-40 (Tab. 2) because the direct precursor GGPP was increased (Verwaal et al. 2007). By adding an additional copy of *crtYB* and *crtI*, the production of carotenoids was increased to 1.947 mg/g DCW in the strain YXWP-47, about two times higher than that of YXWP-40 (Tab. 2). Comprehensive analysis of HPLC results and spectrophotometric measurements demonstrated that nearly pure β-carotene was produced in the yeast cell (SI-8). These results suggested that increasing the expression of rate-limiting enzymes in the pathway could improve the yield of carotenoids effectively. In addition, the accumulation of carotenoids was not found when YXWP-47 was

cultivated in YPD broth (Fig. 4), indicating that the expression of the integrated genes was controlled strictly by the Snf1 signaling pathway and the Gal4p–Gal80p regulatory axis in an anticipated manner.

Using Glucose to Control the Switch Time of the Pathway

In YXWP-47, the carotenoid biosynthetic pathway was induced by galactose. However, upon exhaustion of galactose the integrated pathway would be turned off due to the absence of an inducer. This might go against our efforts to keep the pathway in an open state for the production of valuable biosynthetic products. This problem was solved by disrupting the GAL80 gene in YXWP-47. In the resulting strain, YXWP-53, the regulatory network was changed so that the activation of the *GAL10* and *GAL1* promoters could only be repressed by glucose through the Snf1 signaling pathway. Interestingly, in comparison with YXWP-47, such a change lead to higher accumulation of carotenoids in YXWP-53 when growing on a YPG plate (Fig. 4). The cell growth, glucose consumption and carotenoid accumulation of YXWP-53 were characterized at different growth stages in the shake-flask. It was found that the carotenoids were being produced after the glucose was exhausted, and accumulated continuously until there was no nutrition in medium for sustaining cell growth (Fig. 5A). The production of carotenoids in YXWP-53 reached up to 8.005 mg/g DCW after 72 h of cultivation in YPD broth (Tab. 2, Fig. 5A). Thus, it was expected that by reducing glucose in media and adding a nonfermentable carbon source, the repression of *GAL4* expression could be alleviated and more cellular resources could be devoted to the synthesis of carotenoid.

As anticipated, by varying the ratio of glucose and glycerol in the medium of YXWP-53, the switch time of the assembled pathway was earlier when compared to using glucose as a sole carbon source (Fig. 5B). After 72 hours of fermentation in YPDG with 1% glucose and 1% glycerol as the

carbon sources, the total carotenoid production of 11 mg/g DCW (72.57 mg/L) was achieved in YXWP-53, and the content of β-carotene reached 7.41mg/g DCW (Fig. 6). This yield of total carotenoids reflects a 367-fold increase compared to the strain YXWP-14, which only expressed *crtYB* and *crtI*. To our knowledge, this is the highest level of carotenoid production by an engineered yeast strain (Steinbuchel and Lange 2011; Verwaal et al. 2007). The results also suggested that the introduced pathway could be optimized rationally in a predictable manner by designing a controllable biosynthesis pathway.

Stability of YXWP-53

When the β-carotene biosynthesis genes *crtE*, *crtYB* and *crtI* were expressed by two high copy number plasmids pESC-URA and pESC-LEU in yeast, nearly all the cells lost the ability to produce pigment after ten generations of subculture in YPG medium (data not shown). In contrast, after twenty generations of subculture, there were hardly any white colonies on YPD plates and only slight fluctuations were observed in the ability of YXWP-53 to synthesize β-carotene (SI-9). This showed the strain YXWP-53 was genetically stable during the subsequent subcultures.

Discussion

In previous studies, the "reiterative recombination" (Wingler and Cornish 2011) and "DNA assembler" provided new strategies for construction of biochemical pathways in *S. cerevisiae* (Shao et al. 2009). However, in these two methods, all of constructs in the pathway were integrated into a single locus in the yeast genome. To avoid the problem of recombination caused by repeated sequences, we chose a decentralized assembly strategy.

In this study, multi-gene pathway (six ORF) controlled by *GAL1-GAL10* promoters were integrated into the *S. cerevisiae* genome using pMRI plasmids. The long homologous arm of pMRI

for recombination ensured a higher accuracy for directed integration (Reid et al. 2002). Although the process of basic pMRI construction at the beginning was relatively complex compared to a PCR-based gene integration method (Fang et al. 2011; Shao et al. 2009), the subsequent pMRI plasmid construction was made convenient by varying the promoter, terminator and homologous region according to the general construction scheme (SI-3).

The decentralized assembly strategy also guaranteed the relative stability of the integrated pathway, even though same functional parts were used repeatedly. After 20 generations of subculture, nearly all the colonies of YXWP-53 presented the same color and ability to produce carotennoids. Only one white colony on the YPD plate of the twentieth generation was observed (about 1 out of 800 colonies), which demonstrated that the mutation rate of the strain YXWP-53 was relatively low. We analyzed the genotype of the white mutant, and found that the recombination of the *HMG1* site led to the deletion of the integrated *tHMG1* and *crtE* because of the repeated *tHMG1* region, while *crtYB* and *crtI* on the *HO* and *Ty4* loci were still retained. This also suggested that repeated sequences separated on different sites were more stable than those gathered on single loci.

In a previous study, Yan et al constructed a β-carotene biosynthesis pathway in *S. cerevisiae* in which carotenoid genes were under the control of the constitutive glyceraldehyde 3-phosphate dehydrogenase promoter (GPD promoter) (Yan et al. 2011). However, the maximum accumulation of β-carotene was obtained in the logarithmic phase, and the carotenoid content in dry cell decreased with the culture time. This might be due to the fact that the GPD promoter for the expression of carotenoid genes was only activated at high concentrations of glucose (Partow et al. 2010). In contrast, the expression of the genes in our study was triggered by the GAL4 transcriptional factor, and the switch time of the assembled pathway could be controlled by the

initial concentration of glucose in media. Therefore, carotenoids could be accumulated continually until a steady state was achieved. Such a fermentation pattern would be more suitable for the production of secondary metabolites.

In the shake-flask experiment, compared to using 2% glucose as a carbon source, using 1% glucose and 1% glycerol as the carbon sources increased the production of total carotenoids and β-carotene by 37.4% and 51%, respectively. Originally, we expected an increased glycerol supply (from 1% to 1.5% and 2%) could result in a further improvement of total carotenoid production. However, though a color change was observed earlier for higher glycerol concentrations, the growth rate and total production of carotenoids were actually decreased. We speculated that an early expression of *tHMG1* might have led to an accumulation of IPP, DMAPP, FPP and GGPP (Asadollahi et al. 2010). Nevertheless, since *crtYB* and *crtI* were rate-limiting steps under such a high precursor flux; the excess of precursor was probably not transformed into carotenoids efficiently and resulted in potentially toxic effects on cell (Faulkner et al. 1999; Martin et al. 2003; Scalcinati et al. 2012). Converting the excess of isoprenoid pyrophosphates into corresponding isoprenoid alcohols by dephosphorylation and then secreting them from the cell might mitigate the toxicity of isoprenoid pyrophosphates (Faulkner et al. 1999). However, this mechanism also would led to the loss of the precursors and the decreased the production of carotenoids. Therefore, metabolic flux balance was of great importance when directing the pathway for increased production.

The future of metabolic engineering will most likely lie not only in the assembly of novel pathways, but also in providing these pathways with regulatory systems (Blount et al. 2012; Krivoruchko et al. 2011). In this study, a carbon source-controlled β-carotene biosynthesis pathway was constructed in the yeast genome by using the decentralized assembly strategy, and the highest

reported yield of β-carotene for engineered *S. cerevisiae* was achieved. We believe this strategy would be useful for improving the production of value-added co-products in *S. cerevisiae*.

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List of Figures and Tables

Figure 1. Structure of the pMRI plasmids. Pro = promoter, Ter = terminator, MCS = multiple clone site, HA = homologous arm. The unique cloning site in the MCS and sites marked in the figure can be used for replacement of promoters and terminators.

Figure 2. General scheme of decentralized assembly. Biosynthesis pathway is spilt into several groups according to their function, and then genes with related function are cloned into pMRI plasmids to generate different functional modules. The recycling of *KanMX-pBR322ori* segment is carried out by Cre-mediated recombination. The obtained new strain can be used for the next round of integration.

Figure 3. Schematic of the controllable β-carotene biosynthetic pathway in *S. cerevisiae*. In the *GAL* regulatory system and the Snf1 network, Gal80p, in conjunction with Gal3p and Gal4p, functions as a repressor involved in transcriptional regulation in response to galactose. Mig1p functions as a transcriptional repressor to repress the transcription of *GAL4*. However, Mig1p can be deactivated when cells use galactose as a carbon source, and under these conditions, the *GAL4* gene can be transcribed and expressed as normal. Meanwhile, Gal3p interacts with Gal80p to prevent the inhibition of Gal4p. Gal4p can bind to the *GAL10* and *GAL1* promoters to activate the transcription of downstream genes. After *GAL80* was deleted, the expression of *GAL4* was only controlled by the Snf1 signaling pathway. Consequently, the pathway switch time could be controlled by adjusting the glucose supply. The cells in tubes are YXWP-47 cultured in YPG and YPD broth.

Figure 4. The color change of different strains on YPG agar plate. BY4741 is the host strain in this study. The strains YXWP-14, YXWP-40, YXWP-47 and YXWP-53 were grown 24 h in YPG broth, respectively, then streaked onto a YPG agar plate. After 3 days of incubation at 30°C, the plate was photographed.

Figure 5. Cell growth, glucose consumption and carotenoid accumulation of YXWP-53. (A) YXWP-53 cultivated in YPD medium (2% glucose as carbon source), (B) YXWP-53 cultivated in YPDG medium (1% glucose and 1% glycerol as carbon sources). The cultivations were carried out in 500 ml shake-flasks containing 100ml medium for 72 h. The initial OD_{600} after inoculation was set at 0.05. The error bars represent standard deviation calculated from triplicate experiments.

Figure 6. The production of total carotenoids and β-carotene when YXWP-53 was cultivated in YPDG broth with different ratios of glucose and glycerol for 72 h. Glu = glucose, Gly = glycerol. Total carotenoids in cell extract was measured by absorbance at 450 nm using a spectrophotometer, β-carotene was measured by HPLC. The error bars represent standard deviation calculated from triplicate experiments.

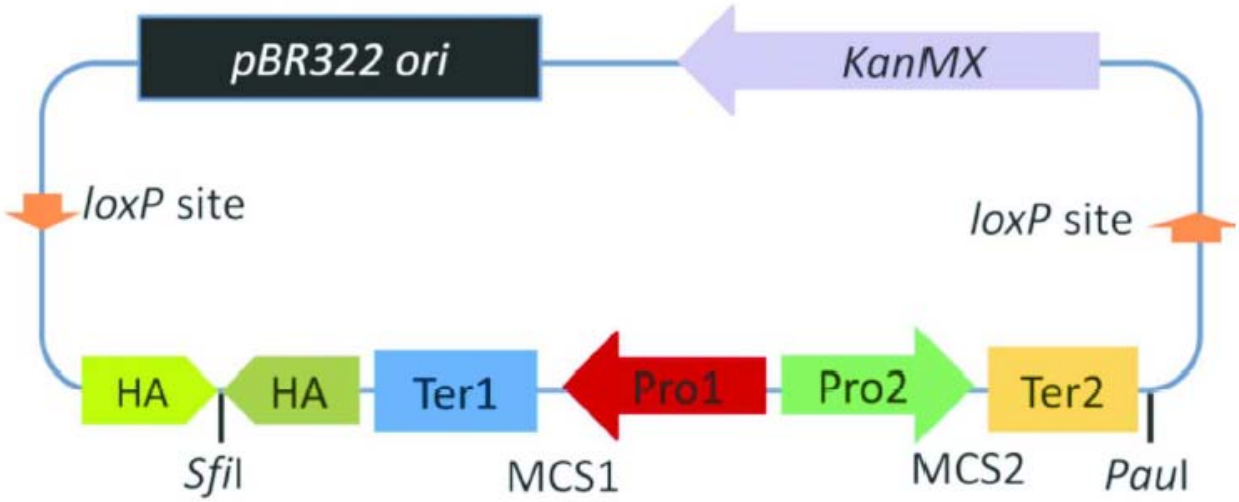
Table1. Functional modules and yeast strains constructed in this study

Functional module* /Strain	Genotype/Description	Reference
pMRI-21-*crtE-tHMG1*	Functional module used to get rid of the rate-limiting step on MVA pathway and FPP to GGPP	This study
pMRI-31-*crtYB-crtI*	Functional module of β-carotene biosynthesis	This study
pMRI-32-*crtYB-crtI*	Functional module of β-carotene biosynthesis	This study
BY4741	*MAT*a, *his3Δ1,leu2Δ0 , met15Δ0, ura3Δ0*	(Brachmann et al. 1998)
YXWP-14	BY4741, $\Delta ho::T_{ADH1}$-*crtYB*-P_{GAL10}-P_{GAL1}-*crtI*-T_{CYC1}	This study
YXWP-40	BY4741, *HMG1*::T_{ADH1}-*crtE*-P_{GAL10}-P_{GAL1}-*tHMG1*-T_{CYC1}, $\Delta ho::T_{ADH1}$-*crtYB*-P_{GAL10}-P_{GAL1}-*crtI*-T_{CYC1}	This study
YXWP-47	BY4741, *HMG1*::T_{ADH1}-*crtE*-P_{GAL10}-P_{GAL1}-*tHMG1*-T_{CYC1}, $\Delta ho::T_{ADH1}$-*crtYB*-P_{GAL10}-P_{GAL1}-*crtI*-T_{CYC1} , $\Delta Ty4::T_{ADH1}$-*crtYB*-P_{GAL10}-P_{GAL1}-*crtI*-T_{CYC1}	This study
YXWP-53	BY4741, *HMG1*::T_{ADH1}-*crtE*-P_{GAL10}-P_{GAL1}-*tHMG1*-T_{CYC1}, $\Delta ho::T_{ADH1}$-*crtYB*-P_{GAL10}-P_{GAL1}-*crtI*-T_{CYC1} , $\Delta Ty4::T_{ADH1}$-*crtYB*-P_{GAL10}-P_{GAL1}-*crtI*-T_{CYC1} $\Delta gal80::URA3$	This study

* The naming convention of the functional module: For example, pMRI-21-*crtE-tHMG1* represents that the pMRI-21 plasmid was used as the backbone, *crtE* inserted into MCS1, and *tHMG1* inserted into MCS2 region.

Table2. The increment of carotenoids production in engineered yeast strains.

Yeast strains	Yield of carotenoids (β-carotene) mg/g DCW	Increased folds	Strategies
BY4741	0	0	The host strain
YXWP-14	0.03 (0.021)	1	Overexpress *crtYB* and *crtI*
YXWP-40	0.959 (0.921)	32	Overexpress *crtE* and *tHMG1*
YXWP-47	1.947 (1.80)	65	Add a copy number of *crtYB* and *crtI*
YXWP-53	8.005 (4.904)	267	Disrupt the *GAL80*，construct a glucose-controlled pathway
YXWP-53	11 (7.406)	367	Adjust the switch time of the pathway



Pro1	Ter1	Pro2	Ter2	HA	plasmid NO
GAL10	ADH1	GAL1	CYC1	-	pMRI-21
GAL10	ADH1	GAL1	CYC1	HO	pMRI-31
GAL10	ADH1	GAL1	CYC1	Ty4	pMRI-32

Figure 1

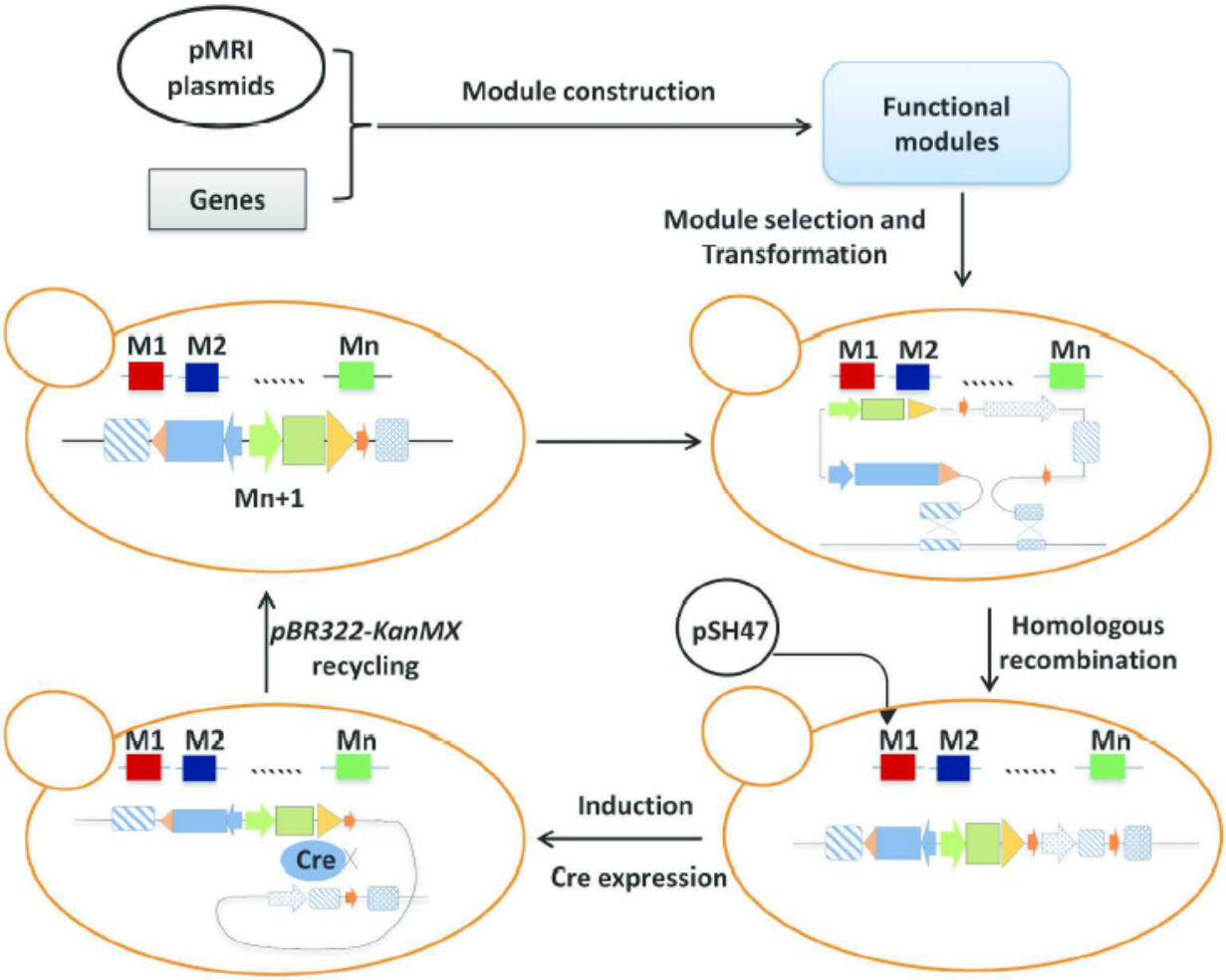


Figure 2

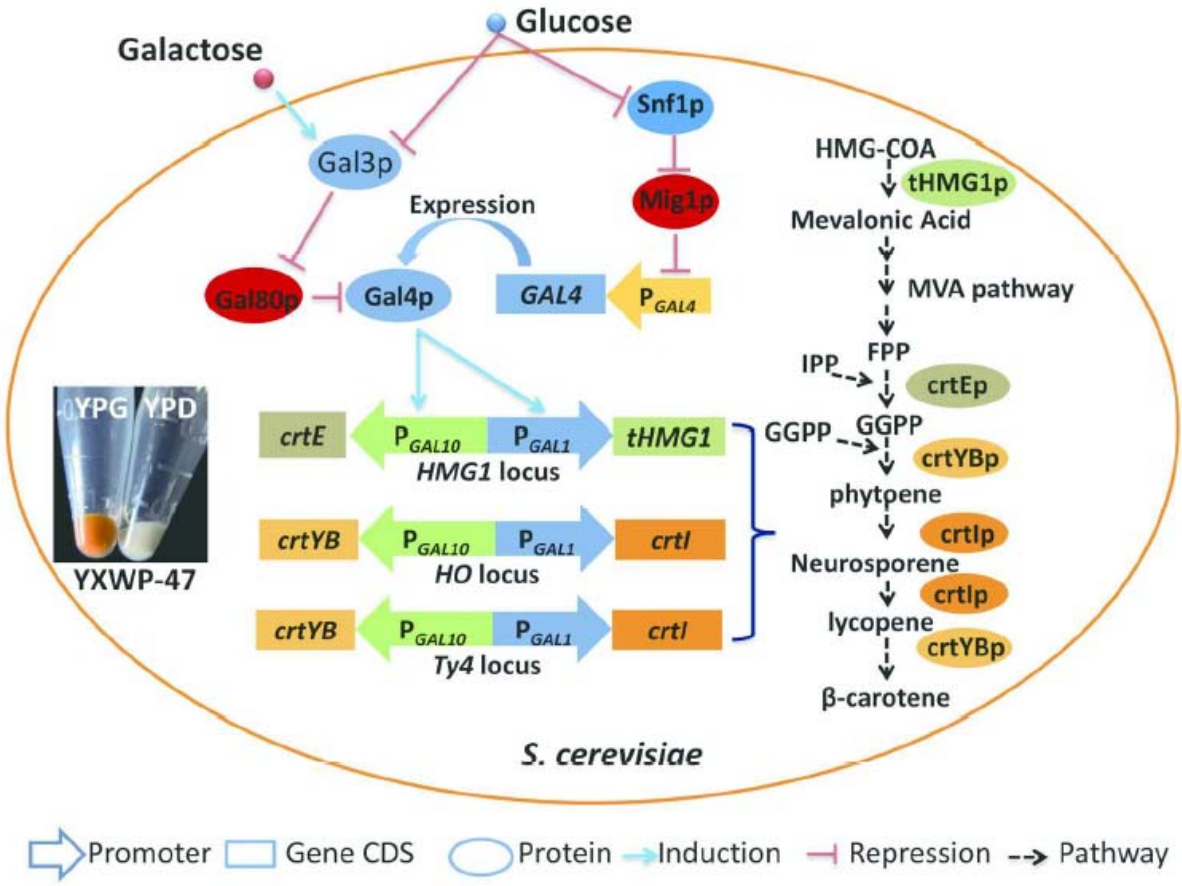
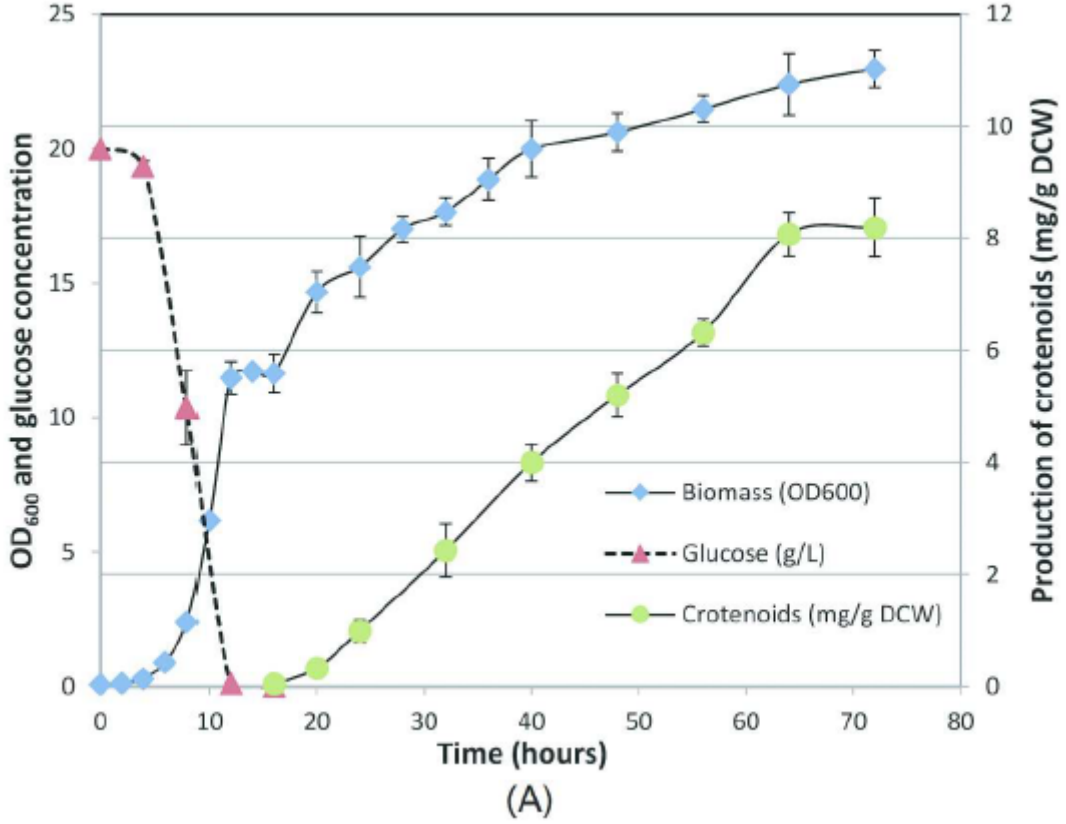


Figure 3

Figure 4



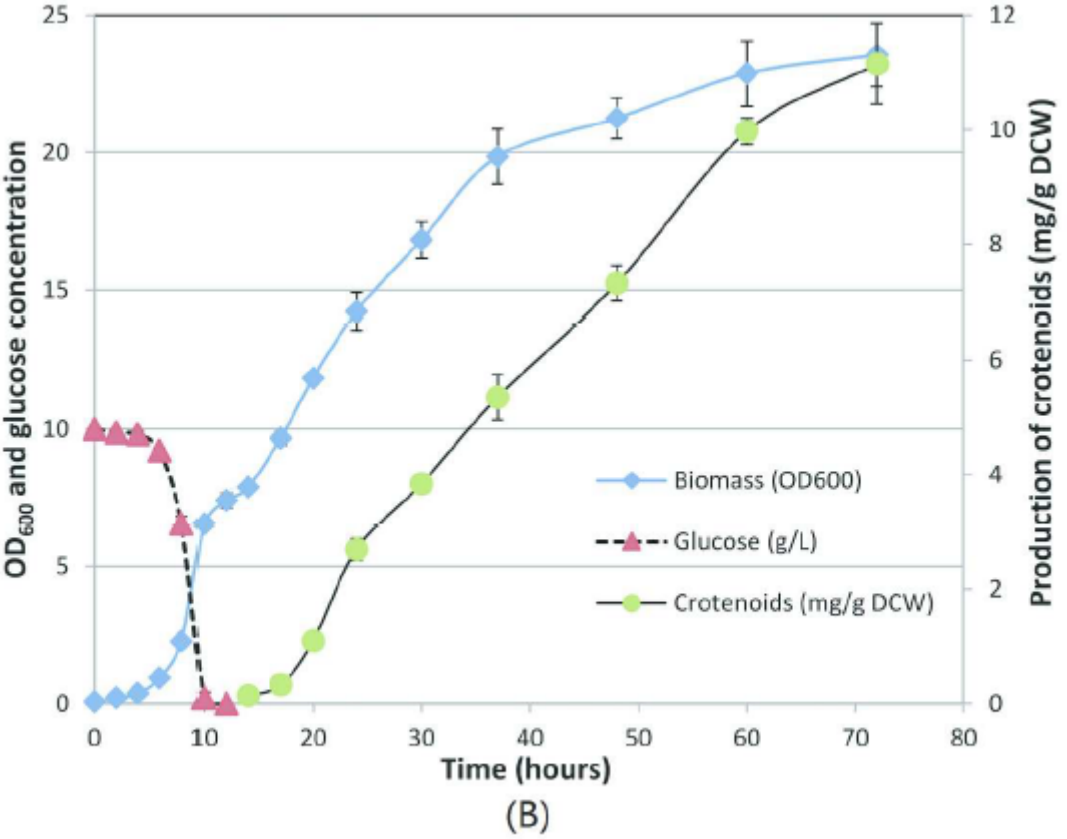


Figure 5

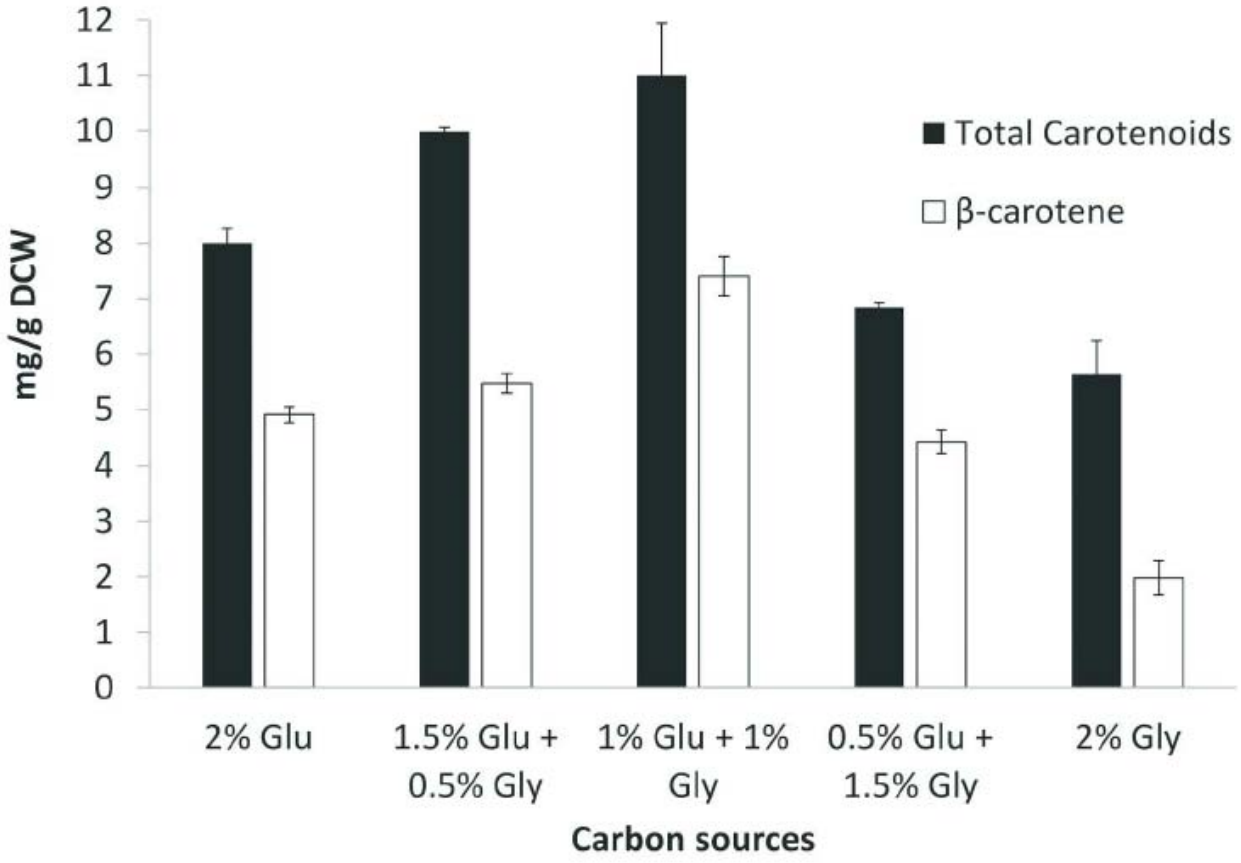


Figure 6