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Combinatorial Engineering of Mevalonate Pathway for Improved Amorpha-4,11-Diene Production in Budding Yeast

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ABSTRACT: Combinatorial genome integration of mevalonate pathway genes was performed with the aim of optimizing the metabolic flux for improved production of terpenoids in budding yeast. In the present study, we developed a novel δ-integration platform to achieve multiple genome integrations through modulating the concentration of antibiotics. By exploiting carotenoid biosynthesis as screening module, we successfully created a library of yeast colonies appeared with various intensities of orange color. As proof-of-concept that carotenoid overproducers could serve to boost the titer of other terpenoids, we further tested engineered strains for the production of amorpha-4,11-diene, an important precursor for antimalarial drug. However, we experienced some limitations of the carotenoid-based screening approach as it was only effective in detecting a small range of pathway activity improvement and further increasing mevalonate pathway activity led to a decreased orange color. By far, we were only able to obtain one mutant strain yielded more than 13-fold amorpha-4,11-diene over parental strains, which was approximately 64 mg/L of caryophyllene equivalents. Further qPCR studies confirmed that *erg10*, *erg13*, *thmg1* and *erg12* involved in mevalonate pathway were overexpressed in this mutant strain. We envision the current δ-integration platform would form the basis of a generalized technique for multiple gene integrations in yeast—a method that would be of significant interest to the metabolic engineering community.

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KEYWORDS: mevalonate pathway; sesquiterpene; carotenoids; combinatorial engineering; pathway activity improvement; *Saccharomyces cerevisiae*

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Introduction

Terpenoids (also called isoprenoids) comprise the largest class of naturally occurring compounds, which can be found in all living organisms (Holstein and Hohl, 2004). Despite the enormous structural diversity, terpenoids are synthesized from two basic C_5 units, isopentenyl pyrophosphate (IPP) and its isomer, dimethylallyl pyrophosphate (DMAPP). In most eukaryotes and some prokaryotes, terpenoids are synthesized through the mevalonate pathway (Lombard and Moreira, 2011). In the initial steps of mevanolate pathway, three acetyl-CoAs were first condensed to form hydroxyl-methyl-glutaryl-CoA (HMG-CoA), which was further reduced to mevalonate by the rate-limiting step of HMG-CoA reductase (HMGR) (Polakowski et al., 1998). Subsequently, phosphorylation and decarboxylation of the mevalonate yielded IPP which in turn produce DMAPP through the action of isomerase. Further condensation of IPP and DMAPP catalyzed by prenyltransferases resulted in geranyl diphosphate (GPP, C_{10}), farnesyl diphosphate (FPP, C_{15}), and geranylgeranyl diphosphate (GGPP, C_{20}). Next, various naturally occurring terpene synthases convert these $C_{10}/C_{15}/C_{20}$ precursors to monoterpenes, sesquiterpenes, and diterpenes, respectively (Tholl, 2006). Further modification by multiple enzymatic steps finally yield active terpenoid derivatives that serve a wide variety of functions such as respiration and electron transport (quinones), hormone signaling (steroids), and antioxidant agents (carotenoids) (Gershenzon and Dudareva, 2007).

Beyond their essential cellular functions, terpenoids have great commercial value to serve as pharmaceuticals (Ajikumar et al., 2010; Anthony et al., 2009; Jiang et al., 2012; Ro et al., 2006) and nutriceuticals (Miura et al., 1998). Traditionally, these valuable terpenoids are extracted from their natural sources but this procedure is laborious and expensive due to the low abundance of these compounds in their natural host (Ishida and Chapman, 2009; Lapkin et al., 2006). Moreover, chemical synthesis of these natural products can be extremely difficult and problematic because of the

structural complexity of terpenoids (Acton and Roth, 1992). Synthetic biology, on the other hand, offers a relatively inexpensive way to produce terpenoids in high quantities. By reconstructing heterologous metabolic pathways in genetically tractable host organisms such as *Saccharomyces cerevisiae*, complex natural products can be produced from inexpensive starting materials by large-scale fermentation processes (Ajikumar et al., 2010; Chang and Keasling, 2006; Westfall et al., 2012).

Although great success has been achieved in the past decade for the overproduction of high-value terpenoids in budding yeast, traditional approach is still a time-consuming and labor-intensive process. In the present study, we sought to develop a novel platform for combinatorial overexpression of mevalonate pathway genes to improve pathway activity. Specifically, the novel δ-integration platform was constructed for simultaneously introducing multiple copies of mevalonate pathway genes into budding yeast genome through modulating the concentration of antibiotics (Fig. 1). The current approach takes advantage of efficient recombination system as well as multiple copies of retrotransposons (Ty) elements of yeast genome (Boeke et al., 1985; Hauber et al., 1985). Thus it is possible to achieve multiple genome integrations by simply applying high concentration of antibiotics, in a similar way as gene duplications demonstrated in *Escherichia coli* (Tyo et al., 2009). In the initial attempt, we tested our novel δ-integration platform for carotenoid production with the aim of harnessing carotenoid-based visual phenotype for screening purpose. However, there were some limitations of the current carotenoid-based approach as it was only effective in detecting a small range of pathway activity improvement and further increasing mevalonate pathway activity led to a decreased orange color. These findings led us to further test another eight yellowish mutant strains for amorpha-4,11-diene production. Among them, one mutant strain yielded more than 13-fold amorpha-4,11-diene over the parental strain, which was approximately 64 mg/L of caryophyllene equivalents.

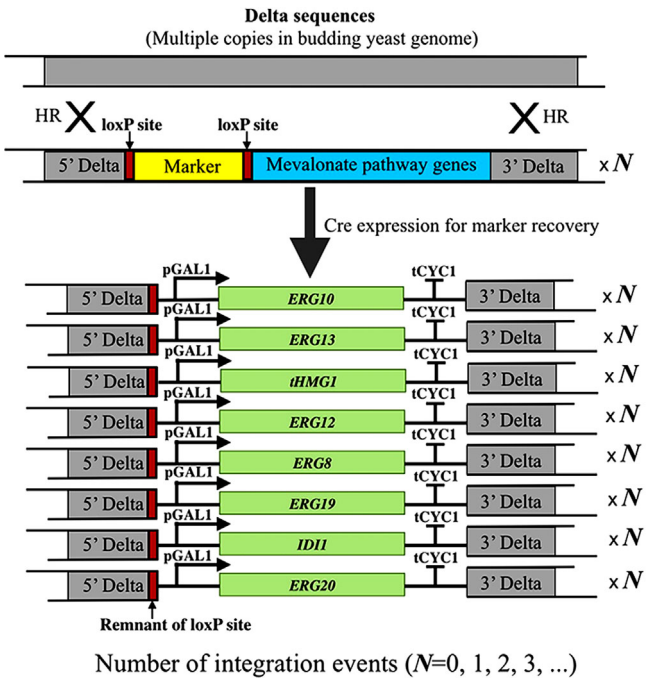

Figure 1. Schematic diagram of δ-integration platform for combinatorial overexpression of mevalonate pathway genes. Cassettes are flanked with homologous sequences to δ-sites to facilitate genome integrations through homologous recombination (HR). Antibiotic selection marker is used to achieve multiple genome integrations by modulating the concentration of antibiotics. Cre recombinase expression can be used for marker recovery so that successive round of combinatorial engineering can be performed.

Materials and Methods

Strains, Plasmids, and Reagents

E. coli strain DH5α was used for general plasmid constructions and the strain was cultivated at 37°C in Luria-Bertani medium with 50 μg/mL ampicillin. *Saccharomyces cerevisiae* strain BY4742 obtained from EUROSCARF was used as the parent strain for all yeast strain constructions. This strain was grown in rich YPD medium. Engineered strains were grown in SD medium with leucine or uracil was dropped out where appropriate. For induction of genes under the control of galactose inducible promoters, *S. cerevisiae* strains were grown in 2% galactose instead of glucose. Plasmid pUG66 used as template for the amplification of antibiotic selection marker was obtained from EUROSCARF. Plasmid pUC18 and pYES2 was purchased from Life Technologies. Plasmid YB/I (YEplac195::TDH3p-crtYB-CYCt; TDH3p-crtI-CYCt) was kindly provided by Prof. Gerhard Sandmann from University of Frankfurt, which was initially constructed by Verwaal et al. (2007). Plasmid pRS425ADS harboring the codon optimized amorpha-4,11-diene synthase gene (Ro et al., 2006) was kindly provided by Prof. Jay Keasling from UC Berkeley. All the chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO). Restriction enzymes, Taq polymerase, alkaline phosphatase (CIP) and T4 ligase were purchased from New England Biolabs (Beverly, MA). iProof HF polymerase and iScript™ Reverse Transcription Supermix were obtained from BioRad (Hercules, CA). Gel extraction kit, PCR purification kit, plasmid purification kit and RNeasy Mini Kit were purchased from Qiagen (Singapore, SG). FastStart Essential DNA Green Master was purchased from Roche, Singapore. Phleomycin was purchased from InvivoGen (San Diego, CA).

Plasmid Construction

Oligonucleotides used for plasmid construction were listed in Table I. To create the platform for multiple-gene integration into yeast genome, novel δ-integration platform was constructed as follows. The delta sequence was amplified from genomic DNA of *S. cerevisiae* BY4742 using primer pair F_Delta_*Hin*dIII and R_Delta_*Eco*RI, digested with *Hin*dIII and *Eco*RI, and inserted into pUC18 cut with the same enzyme pair. The subsequent constructed plasmid was

Table I. Oligonucleotides used for constructing plasmids.

Name	Description
F_Delta_*Hin*dIII	ACCCCAAGCTTTGTTGGAATAAAAATCCACTATC
R_Delta_*Eco*RI	GGAATTCATGGGGGTTCTCTGGAACAG
F_Delta_integ	TGTTGGAATAAAAATCCACTATC
R_Delta_integ	ATGGGGGTTCTCTGGAACAG
F_BLE_SS	ACACGCGTCGACGCATGCATAACTTCGTATAATGTATGCTATACGAAGTTATCCTCGTCCCCGCCGGGTC
R_BLE_*Sal*I	ACACGCGTCGACATAACTTCGTATAGCATACATTATACGAAGTTATCGAGAGCTCGTTTTCGAC
F_pGAL1_*Sph*I	AGAGAGCATGCACGGATTAGAAGCCGCCGAG
R_tCYC1_*Sph*I	AGAGAGCATGCCTTCGAGCGTCCCAAAACCTTC
F_pGAL1Screen	CGTCAAGGAGAAAAAACCCC
R_tCYC1Screen	CTTTTCGGTTAGAGCGGATC
F_ERG10_*Bam*HI	CGGGATCCAAAACAATGTCTCAGAACGTTTACATTG
R_ERG10_*Xho*I	ACACGCTCGAGTCATATCTTTTCAATGACAATAG
F_ERG13_*Bam*HI	CGGGATCCAAAACAATGAAACTCTCAACTAAACTTTG
R_ERG13_*Xho*I	ACACGCTCGAGTTATTTTTTAACATCGTAAGATC
F_tHMG1_*Bam*HI	CGGGATCCAAAACAATGGCTGCAGACCAATTGGTG
R_tHMG1_*Xho*I	ACACGCTCGAGTTAGGATTTAATGCAGGTGAC
F_ERG12_*Bam*HI	CGGGATCCAAAACAATGTCATTACCGTTCTTAAC
R_ERG12_OE	GCTAACAACGGGTCCAAAAGAC
F_ERG12_OE	GTCTTTTGGACCCGTTGTTAGC
R_ERG12_*Xho*I	ACACGCTCGAGTTATGAAGTCCATGGTAAATTC
F_ERG8_*Bam*HI	CGGGATCCAAAACAATGTCAGAGTTGAGAGCC
R_ERG8_*Xho*I	ACACGCTCGAGTTATTTATCAAGATAAGTTTCCG
F_ERG19_*Bam*HI	CGGGATCCAAAACAATGACCGTTTACACAGCATC
R_ERG19_*Xho*I	ACACGCTCGAGTTATTCCTTTGGTAGACCAG
F_IDI1_*Bam*HI	CGGGATCCAAAACAATGACTGCCGACAACAATAG
R_IDI1_*Xho*I	ACACGCTCGAGTTATAGCATTCTATGAATTTG
F_ERG20_*Bam*HI	CGGGATCCAAAACAATGGCTTCAGAAAAAGAAATTAG
R_ERG20_*Xho*I	ACACGCTCGAGCTATTTGCTTCTCTTGTAAAC

named as pδ-BLANK. Next, pδ-BLANK was cut with *Xho*I and dephosphorylated by CIP, inserted with the selection cassette amplified from pUG66 using primer pair F_BLE_SS and R_BLE_*Sal*I to create pδBLE. Next, the pGAL1/tCYC1 cassette was amplified from pYES2 with primer pair F_pGAL1_*Sph*I and R_tCYC1_*Sph*I, digested with *Sph*I and inserted into pδBLE cut with *Sph*I, to yield pδBLE1.1. All eight genes involved in mevalonate pathway were amplified from genomic DNA of *S. cerevisiae* BY4742 with primer pair introducing *Bam*HI and *Xho*I at 5′ and 3′, respectively. Specifically, overlapping-PCR was used to remove unnecessary restriction sites. PCR products were digested with *Bam*HI/*Xho*I, and inserted into pδBLE1.1 cut with the same enzyme pair. Subsequently constructed plasmids were named as pδBLE1.1-ERG10, pδBLE1.1-ERG13, pδBLE1.1-tHMG1, pδBLE1.1-ERG12, pδBLE1.1-ERG8, pδBLE1.1-ERG19, pδBLE1.1-IDI1 and pδBLE1.1-ERG20 (as listed in Table II). To this end, all the mevalonate pathway genes were put under the control of galactose inducible strong promoter. Next, these plasmids were served as template for the PCR amplification of genome integration cassettes using primer pair F_Delta_integ/R_Delta_integ.

Yeast Transformation and Library Construction

To obtain carotenoid-producing yeast strain, standard lithium acetate method was used to introduce plasmid YB/I into *S. cerevisiae* BY4742 and cells were spotted on SD agar plates with uracil dropped out for the selection of successful transformants. For library construction, electroporation was performed as follows. Four hundred microliters of yeast cells together with approximately 20 μg DNA mixture of PCR products with equimolar of integration cassette was electroporated in a 0.2 cm cuvette at 2.5 kV. After electroporation, cells were immediately mixed with 8 mL prewarmed SD-URA medium and incubated for 1 h on a rotary shaker. Following that, cells were collected by centrifugation at 3,000 rpm for 5 min, washed and re-suspended in 2 mL of ddH_2O. Aliquots of 100 μL of cells were spotted on SD-URA plate supplemented with 40 μg/mL of phleomycin.

For the subsequent validation of genome integrations by PCR, large colonies with intense color were randomly picked up from the library and streaked on phleomycin containing plates to eliminate false positive strains. Next, a pair of universal primer F_pGAL1Screen/R_tCYC1Screen was used for the validation of genome integration of mevalonate pathway genes.

Amorpha-4,11-Diene Production in Yeast

For testing amorpha-4,11-diene production in mutant strains, eight mutant strains with intense orange color were re-inoculated on SD + URA medium supplemented with 1 g/L 5′-FOA, subjected to serial dilution and restreaked

Table II. List of plasmids used in the present study.

Plasmid name	Description	Refs.
pUC18		Invitrogen, Singapore
pYES2		Invitrogen, Singapore
pUG66		Gueldener et al. (2002)
YB/I	YEplac195::TDH3p-crtYB-CYC1t; TDH3p-crtI-CYC1t	Verwaal et al. (2007)
pRS425ADS	pRS425::GAL1p-ADS-CYC1t	Ro et al. (2006)
pδ-BLANK	pUC18 derivative containing Delta sequence	This study
pδBLE	pδ-BLANK derivative with BLE selection marker	This study
pδBLE1.1	pδBLE derivative with insertion of GAL1p-CYC1t	This study
pδBLE1.1-ERG10	pδBLE1.1::GAL1p-ERG10-CYC1t	This study
pδBLE1.1-ERG13	pδBLE1.1::GAL1p-ERG13-CYC1t	This study
pδBLE1.1-tHMG1	pδBLE1.1::GAL1p-tHMG1-CYC1t	This study
pδBLE1.1-ERG12	pδBLE1.1::GAL1p-ERG12-CYC1t	This study
pδBLE1.1-ERG8	pδBLE1.1::GAL1p-ERG8-CYC1t	This study
pδBLE1.1-ERG19	pδBLE1.1::GAL1p-ERG19-CYC1t	This study
pδBLE1.1-IDI1	pδBLE1.1::GAL1p-IDI1-CYC1t	This study
pδBLE1.1-ERG20	pδBLE1.1::GAL1p-ERG20-CYC1t	This study

on YPD plate to remove the plasmid YB/I. Single white colonies from YPD plate were inoculated into YPD liquid medium, prepared through conventional lithium acetate approach for subsequent transformation of pRS425ADS. Transformed cells were plated on SD-LEU for the selection of transformants containing pRS425ADS. The parental strain *S. cerevisiae* BY4742 was also transformed with pRS425ADS to serve as control group for the comparison of amorpha-4,11-diene production. Small-scale studies were carried out for amorpha-4,11-diene productions in engineered strains. Specifically, 14 mL sterile tubes containing 3 mL SD medium with leucine dropped out were inoculated to an initial OD_{600} of 0.01 with overnight cultures. All tubes were immediately added with 300 μL dodecane after seeding, to perform two phase fermentation and harvest amorpha-4,11-diene. Both amorpha-4,11-diene production and cell growth profile were continuously monitored for 5 days. Every time, 100 μL of cell culture was taken for measuring OD_{600} by microplate reader (Synergy H1, BioTek, Singapore), and 10 μL dodecane layer was sampled and diluted in 490 μL ethyl acetate for the determination of amorpha-4,11-diene levels by GC-MS. During GC-MS analysis, 1 μL of diluted sample was injected into Shimadzu QP2010Ultra system equipped with a DB5ms column (Agilent Technologies, Singapore). Helium (ultra purity) was used as a carrier gas at a flow rate of 1.0 mL/min. The oven temperature was first kept constant at 80°C for 2 min, and then increased to 190°C at a rate of 5°C/min, and finally increased to 300°C by 20°C/min. Both the injector and mass detector were set at 250°C. Scan mode was used to detect mass range 50–500 *m*/*z*. For the quantitation of amorpha-4,11-diene level, caryophyllene was used for plotting the standard curve.

Intracellular Carotenoid Level Measurement

The carotenoid levels in mutant strains as well as parental strains were measured as follows, with minor modification of the approach described previously (Wang et al., 2009). Single colonies were inoculated into SD-URA medium. Next day, 14 mL sterile round bottom tubes containing 3 mL SD + galactose medium with uracil dropped out were inoculated to an initial OD_{600} of 0.05 with overnight cultures. Two hundred microliters cell culture was used for the extraction of carotenoids as follows. Cells were first harvested by centrifugation at 16,000*g* for 30 s and washed once with 1 mL water. After wash, cells were collected by centrifugation and re-suspended in 200 μL acetone. The re-suspend cell solutions were incubated in dark for 15 min at 55°C with intermittent vortexing. The mixture was centrifuged at 16,000*g* for 30 s and the supernatant carotenoid solutions were transferred to a clean tube, spin dried for 10 min, and re-suspended in 200 μL of hexane for quantification. The absorbance at 450 nm of the extracted carotenoid solutions was measured using microplate reader (Synergy H1, BioTek) to determine the content of carotenoids with color. The relative carotenoid levels were calculated by normalizing to cell densities.

RNA Extraction and Quantitative PCR

Yeast cells were harvested during the exponential growth phase. Approximately 1×10^7 cells were used for the total RNA extraction using RNeasy Mini Kit (Qiagen). Genomic DNA contamination was eliminated by DNase I treatment. RNA concentration was quantified by measuring the absorbance at 260 nm using microplate reader (Synergy H1, BioTek). Next, 500 ng of RNA was converted to cDNA using iScript™ Reverse Transcription Supermix from Biorad.

The gene-specific primers for mevalonate pathway genes and actin (*act1*, internal reference gene) were designed using the ProbeFinder (https://www.roche-applied-science.com), and primers used for qPCR experiment were listed in Table III. The designed PCR amplicons were around 60 bp.

Table III. List of primers used for qPCR experiment.

Name	Length (nt)	T_m (°C)	GC content (%)	Description
F_ACT1_q	18	59	56	tccgtctggattggtggt
R_ACT1_q	22	59	41	tgagatccacatttgttggaag
F_ERG10_q	20	59	55	ccagggttctctatcctcca
R_ERG10_q	19	59	47	aggcgccttttaaagcaac
F_ERG13_q	20	59	45	atcaccgaaactccaaagga
R_ERG13_q	21	60	38	caaatgggcattttctctcaa
F_tHMG1_q	20	59	50	tgccatccatcgaagtaggt
R_tHMG1_q	20	60	50	catggcaccttgtggttcta
F_ERG12_q	21	59	48	gtgtgttggtcaccgagaaat
R_ERG12_q	19	60	53	cacattcacccatggcatc
F_ERG8_q	21	59	52	aggaggatagcgttaccgaac
R_ERG8_q	21	59	38	ttctgtgcgaatgaaaactca
F_ERG19_q	23	59	39	ccgaaaataactttcctacagca
R_ERG19_q	19	59	53	gagaccaatgcagcaaagc
F_IDI1_q	23	59	39	tttccctgatctttggactaaca
R_IDI1_q	21	59	43	cccttcaaacctaattcgtca
F_ERG20_q	20	60	50	ccggtatcacggatgaaaag
R_ERG20_q	21	59	43	ttcacccaatggaatcaagac

Quantitative PCR analysis was performed using LightCycler 96 real-time machine with FastStart Essential DNA Green Master (Roche) according to the manufacturer's instructions. Each 20 μL reaction contained 50 ng of total cDNA, 10 μL FastStart Essential DNA Green Master, 0.5 μM of each primer. Thermal cycling conditions were set as follows: pre-incubation, 1 cycle of 95°C for 10 min; amplification, 45 cycles of 95°C for 10 s, 57°C for 10 s and 72°C for 10 s. To correct for differences in the amounts of starting materials, *act1* was chosen as a reference housekeeping gene. The results were presented as ratios of gene expression between the target gene (gene of interest) and the reference gene, *act1* (Pfaffl, 2001). All assays were performed in triplicate, and the reaction without reverse transcriptase was used as a negative control.

Results

Library Construction for Mutant Strains Producing Carotenoids

We sought to harness carotenoid-based visual phenotype for screening mutant strains with improved mevalonate pathway activity. Previously, lycopene production by introducing carotenogenic genes from the bacterium *Erwinia uredovora* has been widely used in *E. coli* for screening isoprenoid overproducers (Alper et al., 2005; Wang et al., 2009). However, the initial attempt to produce β-carotene in budding yeast by introducing these carotenogenic genes from *E. uredovora* only resulted in quite low β-carotene production levels of approximately 100 μg/g (dry weight) (Yamano et al., 1994). Recently, Verwaal et al. (2007) demonstrated that introducing carotenogenic genes from the carotenoid-producing yeast *Xanthophyllomyces dendrorhous* into *S. cerevisiae* would lead to high-level carotenoid-producing *S. cerevisiae* cells. Based on their findings, introducing *crtYB* (which encodes a bifunctional phytoene synthase and lycopene cyclase) and *crtI* (phytoene desaturase) from the red yeast *X. dendrorhous* to *S. cerevisiae* is sufficient for carotenoid production (as described in Fig. 2) and yeast colonies appear to be yellowish in color. In the present study, we thus chose the plasmid containing *crtYB/I* genes to screen our combinatorial library for mutant strains with increased carotenoid levels.

For library construction, PCR mixture with cassettes harboring mevalonate pathway genes was electroporated into *S. cerevisiae* BY4742 harboring YB/I following the standard electroporation protocol. Noteworthy, the library size could be further increased by adjusting components such as $CaCl_2$, $MgCl_2$, sorbitol, lithium acetate, dithiothreitol (DTT), as well as conditions like electroporation voltage and DNA input, as pointed out previously (Benatuil et al., 2010). As expected, hundreds of large yeast colonies appeared on SD + galactose plates supplemented with phleomycin. As expected, colonies with various degrees of orange color were observed, presumably due to different combinations of mevalonate pathway genes integrated into genome.

For the initial characterization, eight colonies with intense orange color and moderate colony size were picked out from the library and streaked on another galactose-containing plates (supplemented with 40 μg/mL phleomycin) in order to eliminate false positive strains. Since mevalonate pathway

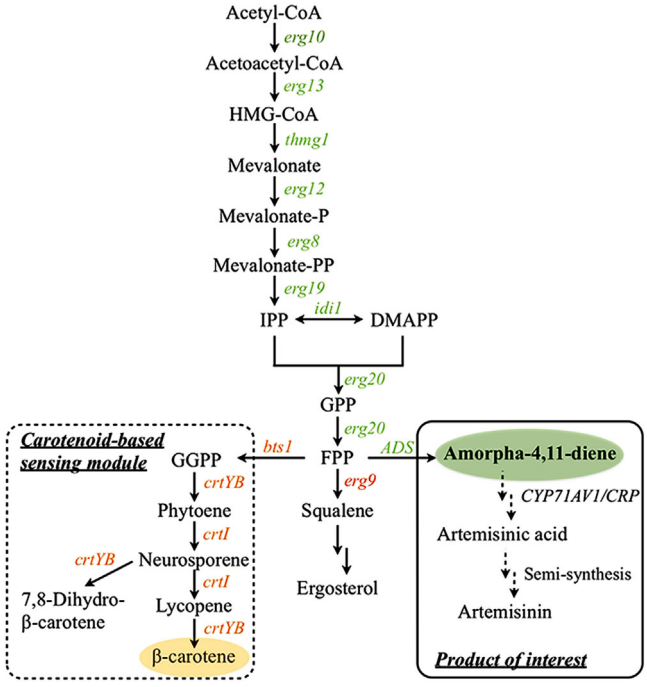

Figure 2. Schematic diagram of mevalonate pathway for carotenoid biosynthesis and amorpha-4,11-diene biosynthesis in budding yeast. Carotenoid-based visual phenotype is used for screening mutant strains with improved mevalonate pathway activity so that carotenoid overproducers can be used for the high-level production of amorpha-4,11-diene, an important precursor for antimalarial drug of artemisinin.

genes were put under control of galactose inducible strong promoter, the parental strain (BY4742::YB/I) and above-mentioned eight mutant strains were also streaked on SD + glucose to evaluate color intensities before induction. As shown in Figure 3, re-streaked strains from library on galactose-containing plate showed more intense color when compared to these on glucose-containing plate.

PCR Validation of Genome Integration Events for Engineered Strains

To further examine whether the cassettes have been successfully integrated into yeast genome, universal primer pair targeting at pGAL1 promoter and tCYC1 terminator region was used for a rough estimation of genome integration events. As shown in Figure S1, gel analysis of PCR products indicated there were successful integrations for mutant strains, but no band was observed for the parental strain. In particular, PCR product from M3 showed a clear three-band pattern (Lane 4), which corresponds to the integration of *idi1*, *erg20* and a third unknown gene. For lane 8 and 9, two-band pattern was observed with the main band close to 1.5 kb and the other weak band near 1.2 kb which was not so obvious from the gel image presented here. For the lane 3, an intense band at 1.0 kb as well as a weak band around 1.5 kb were also observed.

Phleomycin is a glycopeptide antibiotic of the bleomycin family. It binds and intercalates DNA destroying the integrity of the double helix. Previously, chemically induced chromosomal evolution (CIChE) was exploited to evolve the chromosome of *E. coli* with the presence of chloramphenicol (Tyo et al., 2009). Thus we expected that increasing the selection pressure by applying high concentration of phleomycin would facilitate yeast strains with multicopy integration in a similar way. In the present study, we deliberately chose 40 μg/mL of phleomycin during the initial selection step, which is three times higher when compared to conventional approaches for selection of gene deletion events

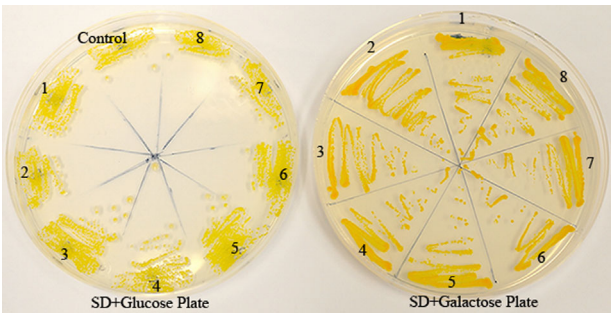

Figure 3. Colors of different carotenoid-producing S. cerevisiae on SD + glucose plate (Left) and SD + galactose plate (Right). Eight individual transformants from the library were streaked onto SD agar plates supplemented with glucose and galactose as carbon sources, respectively. For the glucose-containing plate, the parental strain *S. cerevisiae* harboring plasmid YB/I was streaked for the comparison of color intensity.

(Gueldener et al., 2002). As can be seen from Figure S2, further PCR verification of the mutant library confirmed there was a clear trend of multiple integration events for larger colonies (Lane 1–5) and potentially single integration for smaller ones (Lane 6–8).

Investigation of Amorpha-4,11-Diene Production in Carotenoid Overproducers

As proof-of-concept that mutant strains obtained from library could serve for high-level production of other terpenoids, we further tested these mutant strains for the production of amorpha-4,11-diene, an important precursor for antimalarial drug artimisinin (Fig. 2; Ro et al., 2006). To simplify the procedure, we did not perform marker removal for successive rounds of combinatorial engineering. However, it is highly possible that marker removal to avoid unnecessary robbing of intracellular building blocks such as nucleotides and amino acids, and successive rounds of combinatorial engineering to integrate more mevalonate pathway genes might be able to further boost the product titer.

Specifically, eight mutant strains (same strains as shown in Fig. 3) were transformed with plasmid pRS425ADS, which harbors amorpha-4,11-diene synthase gene (*ADS*) from *A. annua* for the conversion of FPP to amorpha-4,11-diene (Ro et al., 2006). During the evaluation of amorpha-4,11-diene production, mutant strains showed similar growth profiles to parental yeast strains; however, there was a significant improvement of amorpha-4,11-diene levels for all mutant strains (Fig. 4). After normalized to cell density, the best producers (M7 and M8) were able to produce 6-fold of amorpha-4,11-diene over parental strains after 5 days cultivation, which is very impressive considering that only one round of combinatorial engineering was performed at this moment. Based on the PCR validation result as shown in Figure S1, the high level of amorpha-4,11-diene for M7/M8 might be attributed to the integration of *thmg1* encoding the rating limiting step, which is consistent with the previous findings (Polakowski et al., 1998; Ro et al., 2006). On the other hand, the integration of other mevalonate pathway genes without *thmg1* showed only a moderate improvement in amorpha-4,11-diene level. In particular, M3 only produced twofold of amorpha-4,11-diene over parental strains even though three genes were found to be integrated into genome (Fig. S1).

Since the initial test showed all eight mutant strains produced higher level of amorpha-4,11-diene over parental strains, we further examined the correlation of amorpha-4,11-diene levels and carotenoid levels to confirm the effectiveness of current carotenoid-based screening approach. Out of our expectation, the color intensity for M7/M8 appeared to be yellowish when cultured in liquid medium supplemented with galactose as carbon source, although M7/M8 showed the highest absorbance at 450 nm (data not shown). Previously, Verwaal et al. (2007) showed increasing orange color intensities were observed when *BTS1*

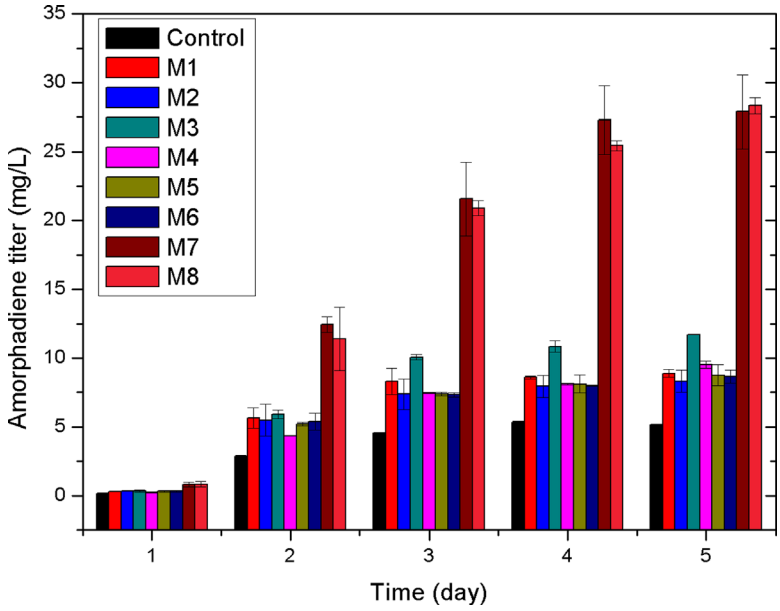

Figure 4. Time course of amorph-4,11-diene production in *S. cerevisiae* mutant strains and parental strains harboring pRS425ADS. Amorpha-4,11-diene levels were continuously measured for 5 days.

and *crtE* encoding GGPP synthase were overexpressed. In particular, accumulation of the intermediate of lycopene was detected for strains with the episomal vector containing *crtYB*/*crtI*/*crtE*. However, the same study also showed that there was a clear color transition from orange to yellow when coupled with additional overexpression of the catalytic domain of HMGR encoding the rate-limiting step of mevalonate pathway, albeit there was an approximate sevenfold improvement of total carotenoid levels after introducing additional copy of the truncated HMGR (Verwaal et al., 2007). It is highly possible that the increasing orange color intensity for engineered yeast strains in their findings might correlate with the accumulation of red color pigment of lycopene instead of β-carotene and other carotenoids. However, it is still not clear why introducing GGPPS synthase to increase the flux toward GGPP would lead to the accumulation of intermediate lycopene, but the improvement of the mevalonate pathway activity by over-expressing the truncated HMGR could lead to a decreased orange color. One hypothesis is that increasing level of mevalonate pathway activity would change the membrane fluidity through the manner of accumulation of ergosterols, which would facilitate the CrtYB-mediated conversion of lycopene to β-carotene (Fig. 2). To further test whether these mutant strains with yellowish appearance could yield high levels of amorpha-4,11-diene, we randomly picked another eight yellowish large colonies for characterizing amorpha-4,11-diene production. As shown in Figure 5, among them, there were two mutant strains produced significantly higher levels of amorpha-4,11-diene and the best producer (M3-2nd) from this batch could even yield more than 13-fold of amorpha-4,11-diene over parental strains, which was approximately of 64 mg/L of caryophyllene equivalents. Further qPCR validation of gene expression levels confirmed that mRNA levels of *erg10*, *erg13*, *thmg1*, and *erg12* for M3-2nd (as shown in Fig. 5) were significantly higher over parental strains (Fig. 6). Moreover, the melting curve analysis confirmed there were unique peaks for all reactions and no primer dimer peak was observed (Fig. S3). Based on these findings, it seems further modification of carotenoid biosynthesis module is needed to provide reliable screening results. To our knowledge, use centromeric plasmid instead of episomal plasmid and exploit lycopene as the end-product instead of β-carotene should be more desirable for screening purpose, which will be reported in due course.

Discussion

Our approach harnesses the efficient recombination system of budding yeast, which has been widely used for assembling synthetic pathway and bacterial genome (Gibson et al., 2010; Shao et al., 2009). In the present study, our new δ-integration platform takes the advantage of antibiotic selection marker for the selection of multiple genome integrations. Conventional approach requires several rounds of genome integration with different selection markers to introduce several genes into genome and it is a trial-and-error process to achieve the best combinations (Farhi et al., 2011; Ro et al., 2006; Scalcinati et al., 2012; Westfall et al., 2012). For example, traditional δ-integration platform that relies on auxotrophic selection marker often results in single integration into yeast chromosome (Lee and Da

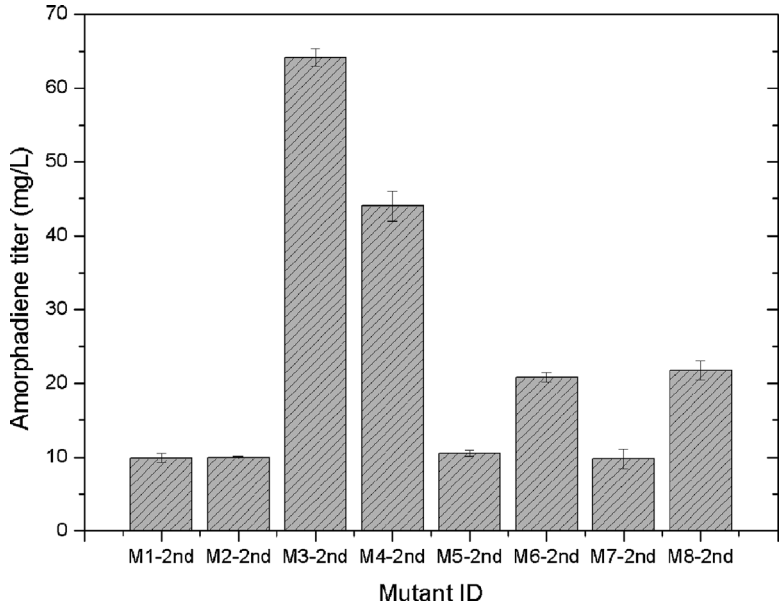

Figure 5. Amorpha-4,11-diene production of mutant *S. cerevisiae* strains obtained from carotenoid producing library with large colony size and yellowish appearance. Amorpha-4,11-diene levels were measured after 5 days. Data represented the average and standard deviation of three independent experiments.

Sliva, 1997). Anther drawback of traditional δ-integration is the pop-out issue for sequential integrations as the chance of replacing previously inserted genes is high when the same promoter and terminator are used for subsequent genes. Recently, Westfall et al. (2012) integrated all mevalonate pathway genes into yeast genome using four different auxotrophic selection markers. And the same group almost depleted all available selection markers to construct yeast strains for high-level production of artimisinic acid (Paddon et al., 2013). However, it is possible for our novel δ-integration platform to integrate all mevalonate pathway genes into genome within one round—simply using dual promoter system and applying higher selection pressure, but our approach will not deplete selection markers for the future use.

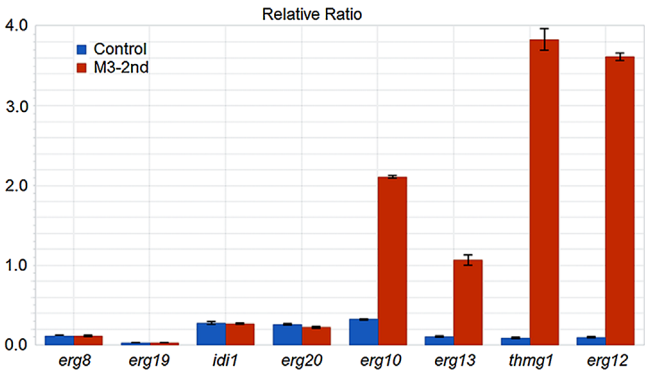

Figure 6. qPCR studies to determine overexpression of mevalonate pathway genes. Two cultures of each strain, parental strain *S. cerevisiae* BY4742, M3-2nd, were inoculated in SD medium with 2% galactose at initial OD600 of 0.05 and harvested after 12 h for qPCR studies. Data represented the relative abundances of mevalonate pathway genes in each strain with respect to that of act1, which encodes actin and serves as an internal control. Data represented the average and standard deviation of three independent experiments.

Combinatorial engineering is a powerful method to achieve balanced metabolic flux and improved product yield (Ajikumar et al., 2010; Pfleger et al., 2006; Wang et al., 2009). For example, high-level production of taxadiene was achieved by multivariate search of different combinations of promoter strength and plasmid copy number for expressing taxadiene metabolic pathway (Ajikumar et al., 2010). In the present study, we were able to achieve beneficial combinations with higher pathway activity in a single round through combinatorial overexpression of mevalonate pathway genes. Due to some limitations of carotenoid-based visual screening approach, the best producer obtained at this moment only produced 13-fold of amorpha-4,11-diene over parental strains, which was approximately 64 mg/L of caryophyllene equivalents. Thus future work will focus on developing a more reliable sensing device so that mutant strains with higher pathway activities can be easily screened out from the library. Considering great improvement of amorpha-4,11-diene level was achieved when all mevalonate pathway genes were overexpressed (Westfall et al., 2012), multiple cycles of combinatorial engineering to incorporate more mevalonate pathway genes into genome is expected to further improve product titer. Specifically, *loxP* site is deliberately introduced at 5′ and 3′ end of selection marker during the initial design of platform to facilitate Cre-mediated marker removal (as

shown in Fig. 1), so that successive rounds of combinatorial engineering can be performed in a similar way as traditional approach for multiple gene knockouts in budding yeast (Gueldener et al., 2002). Apart from overexpression of rate-limiting enzymatic step to increase pathway activity, there are various other approaches that could further boost product titer, namely, knockout/tune-down of competing pathway, protein engineering of rate-limiting enzymes, manipulation of promoter strength as well as cofactor balance. Take cofactor engineering as an example, manipulating cofactor balance such as NADPH/NADH showed great promise to improve the yield of isoprenoid derivatives as well as products involved in other metabolic pathway (Johannes et al., 2007; Ma et al., 2011; Scalcinati et al., 2012). Since HMGR catalyzed enzymatic step is the rate-limiting step in mevalonate pathway and this enzyme utilizes NADPH as cofactor, further incorporation of cofactor regeneration system to increase the supply of NADPH is expected to improve catalytic efficiency of HMGR-mediated step and increase the product titer further. In the near future, we will exploit the possibility of combinatorial engineering approach to optimize other metabolic pathways for producing other high-value therapeutics as well as biofuels.

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