Refactoring β-Amyrin Synthesis in *Saccharomyces cerevisiae*

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Triterpenoids are a highly diverse group of natural products and used particularly as medicine. Here, a strategy combining stepwise metabolic engineering and transcriptional control was developed to strengthen triterpenoid biosynthesis in Saccharomyces cerevisiae. *Consequently, an efficient biosynthetic pathway for producing β-amyrin, a commercially valuable compound and precursor of triterpenoids, was constructed through expressing a plant-derived β-amyrin synthase. Introducing a heterologous squalene monooxygenase greatly dragged intermediate metabolite squalene toward β-amyrin. Increasing squalene pool by overexpressing IPP isomerase, FPP, and squalene synthase further enhanced β-amyrin synthesis of 49-folds. Through reconstructing the promoters with the binding site of transcription factor UPC2, directed transcriptional regulation on engineered pathway was availably achieved, resulting in β-amyrin titer increased by 65-folds. Using ethanol fed-batch fermentation, β-amyrin titer was finally improved up to 138.80 mg/L with a yield of 16.30 mg/g dry cell, almost 185 and 232 and folds of the initially engineered strain, respectively.* © 2015 American Institute of Chemical Engineers *AIChE J*, 61: 3172–3179, 2015
Keywords: β-amyrin, metabolic engineering, transcriptional regulation, triterpenoid, *Saccharomyces cerevisiae*

Introduction

Triterpenoids are a highly diverse group of natural products widely distributed in plants and used in wide-range fields particularly as medicine because of their pharmacological effects.[1,2] For example, glycyrrhizin, a triterpenoid extracted from licorice was applied worldwide medicinally due to various pharmacological activities[3,4] and used as a natural sweetener because of its 150 times higher sweetness than sucrose.[3,5] A key precursor of triterpenoids has been verified as β-amyrin,[6,7] which particularly reflects the potential anti-inflammatory, anti-hyperglycemic, and hypolipidemic effects besides the pharmacological activities similar as glycyrrhizin.[8,9] β-amyrin can be extracted with nonpolar solvent from plant with very low yield, that is, the highest content found in dandelion is only 5 mg/g dry material, resulting in its complicated and costly purification procedure generally including supercritical fluid extraction/adsorption, decolorization, and multistep chromatographic separation, which makes the high price of β-amyrin about $2500 per gram.[10] In addition, the extraction of β-amyrin from the natural sources can be time consuming, wasteful on the natural resources and even destructive to plant communities.[11] The scarce supply from plant and high price greatly hinder the widespread use of β-amyrin, so the development of a promising biological process for β-amyrin production would diversify its supply chain.

Progress in metabolic engineering and synthetic biology has enabled to engineer microbes for efficiently synthesizing natural products. The high yield production of several terpenoids has been achieved using effective metabolic engineering strategies such as overexpressing targeted enzymes, upregulating the transcriptional activator UPC2 (a global transcription factor regulating the sterol synthesis in *Saccharomyces cerevisiae*) to improve the flow of terpenoid biosynthetic pathway.[12,13] The well-known and most inspiring case is the production of artemisinic acid in the engineered yeast by genetically manipulating the biosynthetic machinery.[12] The development of new tools such as rapid assembly of pathways and identification of new pathways have also provided the opportunity for engineering yeast cells to produce β-amyrin.

Biosynthesis of β-amyrin is actually derived from mevalonic acid (MVA) pathway (Figure 1). To date, several β-amyrin synthases have been identified from various plants,[14,15] but there have been few efforts to engineer *S. cerevisiae* for production of β-amyrin. Kirby et al.[16] ever achieved β-amyrin production in yeast with a titer of 6 mg/L, but no following work about improvement was reported. In this study, aim is to largely increase the productivity of β-amyrin using metabolic engineering strategies in *S. cerevisiae*. To do this, we sought to construct an efficient and scalable *S. cerevisiae* for

Additional Supporting Information may be found in the online version of this article.

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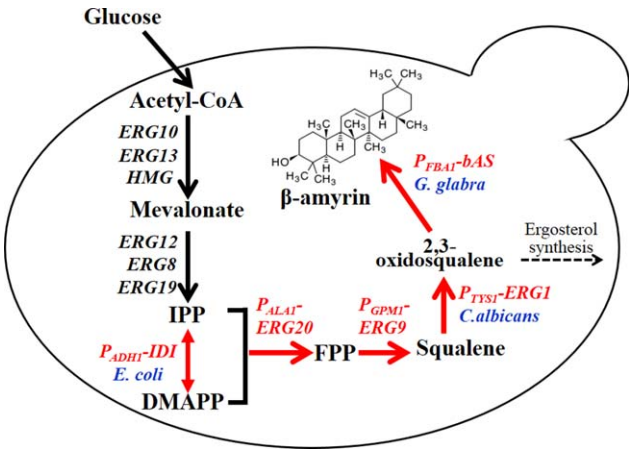

Figure 1. Production of β-amyrin via mevalonate pathway.

Bold and red arrows represent engineered reactions. The gene *bAS* encoding β-amyrin synthase from *G. glabra* is expressed under P_{FBA1} promoter. Genes for isopentenyl pyrophosphate isomerase (*IDI*) and 2,3-oxidosqualene synthase (*ERG1*), respectively, cloned from *Escherichia coli* and *C. albicans* are induced by P_{ADH1} and P_{TYS1} promoters. *ERG20* and *ERG9* genes encoding farnesyl pyrophosphate (FPP) and squalene synthase amplified from *S. cerevisiae* are controlled by P_{ALA1} and P_{GPM1} promoters, isopentenyl pyrophosphate (IPP), dimethylallyl diphosphate (DMAPP). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

overproduction of β-amyrin by combined stepwise rational refactoring and a directed flux regulation strategy. Initially, *S. cerevisiae* was engineered to produce β-amyrin through introducing a β-amyrin synthase gene (*bAS*) from *Glycyrrhiza glabra*. In order to increase the supply of precursor 2,3-oxidosqualene for β-amyrin synthesis, one of main regulatory steps was strengthened by introducing a heterogenous squalene monooxygenase from *Candida albicans*. The metabolites of the upstream MVA pathway were drawn to β-amyrin via overexpressing key enzymes. Moreover, flux for β-amyrin biosynthesis was further upregulated at transcriptional level through implanting the binding site responsible for UPC2 at the upstream of each hired promoter. This study reflects the availability of transcriptional regulation in pathway engineering of yeast. Believably, this engineered yeast would serve as a promising host for further production of various triterpenoids derived from β-amyrin.

Materials and Methods

Strains, media, and cell cultivation

S. cerevisiae INVSc1 (MATa/MATα his3△1 leu2 trp1-289 ura3-52) was used as host strain. Yeasts were maintained on YPD plates containing 10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose, and 20 g/L agar. Engineered yeasts carrying pRS41H or pRS42K plasmids (EUROSCARF, Frankfurt, Germany, Supporting Information, Figure S1) were selected on YPD plates containing 500 mg/L hygromycin B (Merck, Darmstadt, Germany) or 200 mg/L G418 (Genview, FL), respectively. Strains containing hphNT1 or KanMX selection markers were selected on YPD plates containing 500 mg/L hygromycin B or 200 mg/L G418. For shake-flask cultivation, engineered yeasts were cultured in YPD liquid medium with corresponding antibiotics when necessary. The cultures were incubated at 30°C and 170 rpm for aerobic growth. Fed-batch fermentations were carried out in well-controlled 5-L bioreactor (Minifors, Switzerland) with a working volume of 3 L. The temperature was kept constant at 30°C. The air flow was 3 L/min (1 vvm). Bioreactor was inoculated to an initial OD_{600} of 0.20. The optical density at 600 nm was determined using a spectrophotometer, model U-2900 (HITACHI, Japan). The fitting curve between dry cell weight (y, g/L) and OD_{600} (x) is $y = 0.4444x - 0.2187$.

Plasmids and strains construction

β-amyrin synthase gene (accession no. AB037203) derived from *Glycyrrhiza glabra* was chemically synthesized after codon optimization (http://www.jcat.de). Isopentenyl pyrophosphate isomerase (*IDI*) from *E. coli* and squalene monooxygenase (*ERG1*) genes from *C. albicans* were obtained by polymerase chain reaction (PCR). The genes *ERG20* and *ERG9* encoding FPP synthase and squalene synthase were cloned from *S. cerevisiae*. The individual gene expression cassette as promoter–gene–terminator was assembled by overlap extension PCR. Yeast promoters (P_{FBA1}, P_{TYS1}, P_{GPM1}, P_{ALA1}, P_{ADH1}) and terminators (T_{FBA1}, T_{TYS1}, T_{GPM1}, T_{ALA1}, T_{ADH1}) were PCR-amplified from the genomic DNA of *S. cerevisiae* INVSc1. All primers used are shown in Tables S1–S3, Supporting Information.

The β-amyrin producing strains were constructed via homologous recombination mediated DNA assembler.[17] Each cassette was designed with flanking overlap homology sequences that can direct assembly into a plasmid or integration into a chromosomal sequence by yeast recombination machinery.[17] Plasmid-based strains SpHb and SpKb were constructed by cotransforming gene expression cassette P_{FBA1}-*bAS*-T_{FBA1} and the linearized single copy pRS41H or multicopy pRS42K plasmids into *S. cerevisiae*. SpHsb or SpKsb strains were engineered via cotransforming P_{FBA1}-*bAS*-T_{FBA1}, P_{TYS1}-*ERG1*-T_{TYS1} expression cassettes together with linearized pRS41H and pRS42K, sequentially. SpHib strain resulted after transformation of P_{FBA1}-*bAS*-T_{FBA1}, P_{TYS1}-*ERG1*-T_{TYS1}, P_{GPM1}-*ERG9*-T_{GPM1}, P_{ALA1}-*ERG20*-T_{ALA1}, P_{ADH1}-*IDI*-T_{ADH1} cassettes and linearized pRS41H. For genomic integration of β-amyrin biosynthesis pathway, the rDNA locus on chromosome XII was used. SGsb and SGib strains were constructed similarly as SpHsb and SpHib, additional selection marker cassette P_{TEF1}-*hphNT1*-T_{CYC1} were cotransformed and integrated into rDNA locus. SGibS was produced from SGib by introducing a 50-bp sequence including 7-bp binding site responsible for UPC2 at the upstream of promoters. The strength of promoter was assayed using eGFP as reporter by flow cytometry (Becton-Dickinson, America). The genotypes of strains are shown in Figure 2. The detailed experimental process and primers are shown in Supporting Information methods 1.1, 1.2, and Table S4, respectively.

Quantitative PCR

Fresh samples of cell cultures at 36 h were used to determine the relative expression ratio of genes via quantitative PCR (qPCR). The total RNA was isolated using Yeast RNA Kit (OMEGA, Doraville, GA) following the manufacturer's instructions. RNA concentration was quantified by measuring the absorbance at 260 nm using NanoDrop 2000c (Thermo Scientific, Waltham, MA). Five hundred nanogram of RNA was then reversed transcribed into cDNA using Transcriptor First Strand cDNA Synthesis kit (Roche, Indianapolis, IN).

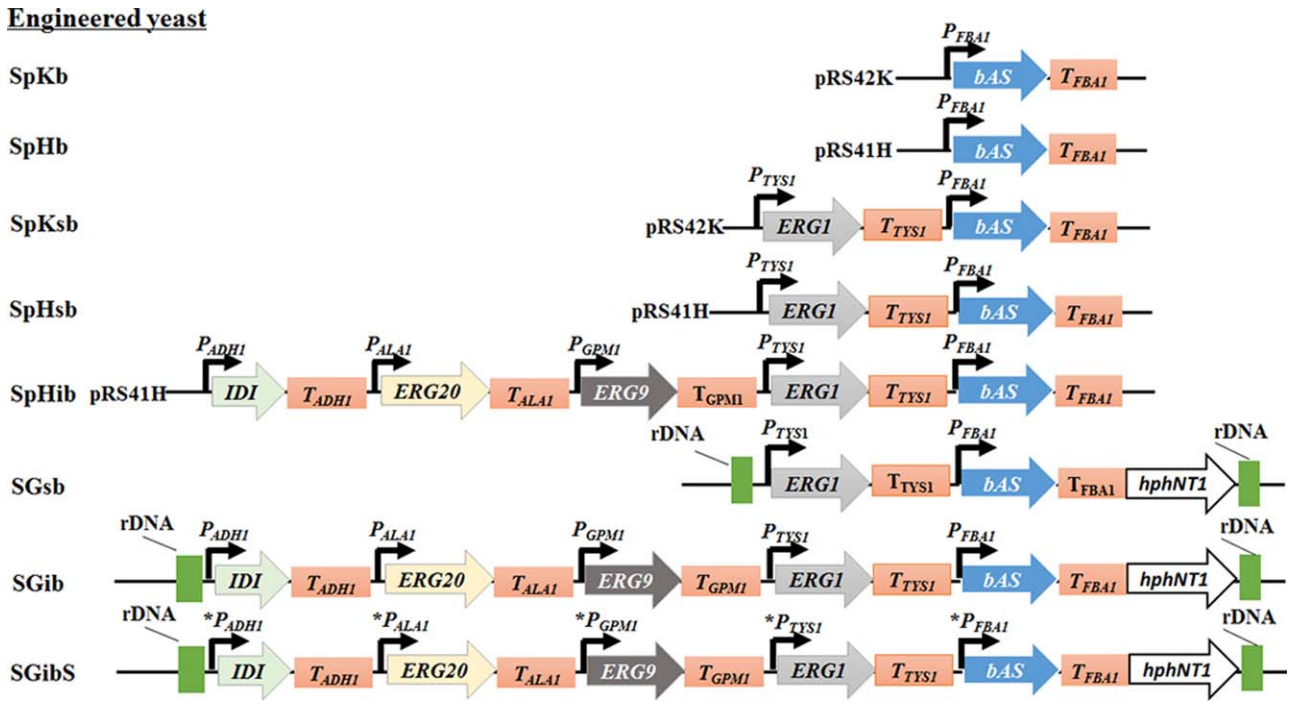

Figure 2. Construction strategy of plasmid-based and genome-integrated strains.

$*P_{FBA1}$, $*P_{TYS1}$, $*P_{GPM1}$, $*P_{ALA1}$, and $*P_{ADH1}$ stand for the promoters refactored sterol regulatory element responsible for binding UPC2. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

The qPCR analysis was carried out using the housekeeping gene *ACT1* as a reference gene at the LightCycler® 96 system (Roche) using LightCycler SYBR Green I Master Kit (Roche) following the manufacturer's instructions.[18] Primers used for qPCR are shown in Table S5, Supporting Information.

Extraction, identification, and quantitation of β*-amyrin and metabolites*

β-Amyrin production during the fermentation course was determined as described previously.[16] Yeast culture (40 mL) was centrifuged for 5 min at 3000*g* to pellet cells. The cells were resuspended in 10 mL of a fresh solution of 20% (w/v) KOH in 50% ethanol and boiled for 10 min to cell lysis. After cooling, *β*-amyrin was extracted using 10 mL of hexane with vortexing for 5 min. This procedure was repeated twice. The hexane phase was rotary evaporated for 1 min, and the concentrated product was resuspended in 1 mL of hexane. For gas chromatography/mass spectrometry (GC/MS) analysis, the extracts were trimethylsilylated with 50 *μ*L of *N*-methyl-*N* (trimethyl sylil) trifluoro acetamide (Sigma-Aldrich) for 30 min at 80°C.[19] GC/MS was performed using a GCMS-QP2010 Plus (Shimadzu) with a DB-5MS column (Agilent). The carrier gas was He with the flow rate of 1.2 mL/min. The injection temperature was 300°C with a 30:1 split ratio. The oven temperature was held at 80°C for 1 min after injection, and was then ramped to 280°C at 20°C/min, held at 280°C for 15 min, ramped to 300°C at 20°C/min and finally held at 300°C for 5 min. Full mass spectra were generated for metabolite identification by scanning within the *m*/*z* range of 40–440. Peaks were identified by comparing the retention time and mass spectra with those of the authentic standards. For quantification of metabolites including *β*-amyrin, squalene, and ergosterol, samples were run in same column condition using a GC2010 (Shimadzu) with a TG-5MS column (Thermo).

Results

Construction and activation of β*-amyrin pathway in* S. cerevisiae

The prerequisite precursors for *β*-amyrin production were available within wild *S. cerevisiae*, therefore, initially *bAS* downstream of P_{FBA1} was expressed in multicopy plasmid pRS42K and dubbed as SpKb. The qPCR showed the effective transcription of *bAS* gene in SpKb (Figure 3). After cultivation in a batch for 5 days, *β*-amyrin production of SpKb was confirmed via GC/MS (Figure 4). SpKb strain was able to synthesize a low amount of 0.75 mg/L *β*-amyrin with a yield of 0.07 mg/g dry cell and productivity of 0.01 mg/(L·h). When expressing *bAS* under the same promoter as SpKb in single copy plasmid pRS41H, engineered SpHb strain produced

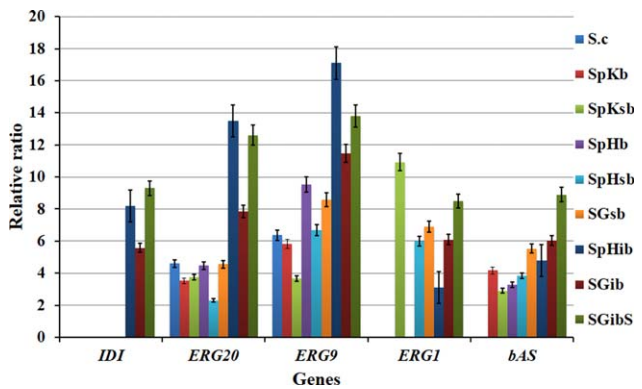

Figure 3. Transcriptional quantification of genes via qPCR.

[Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

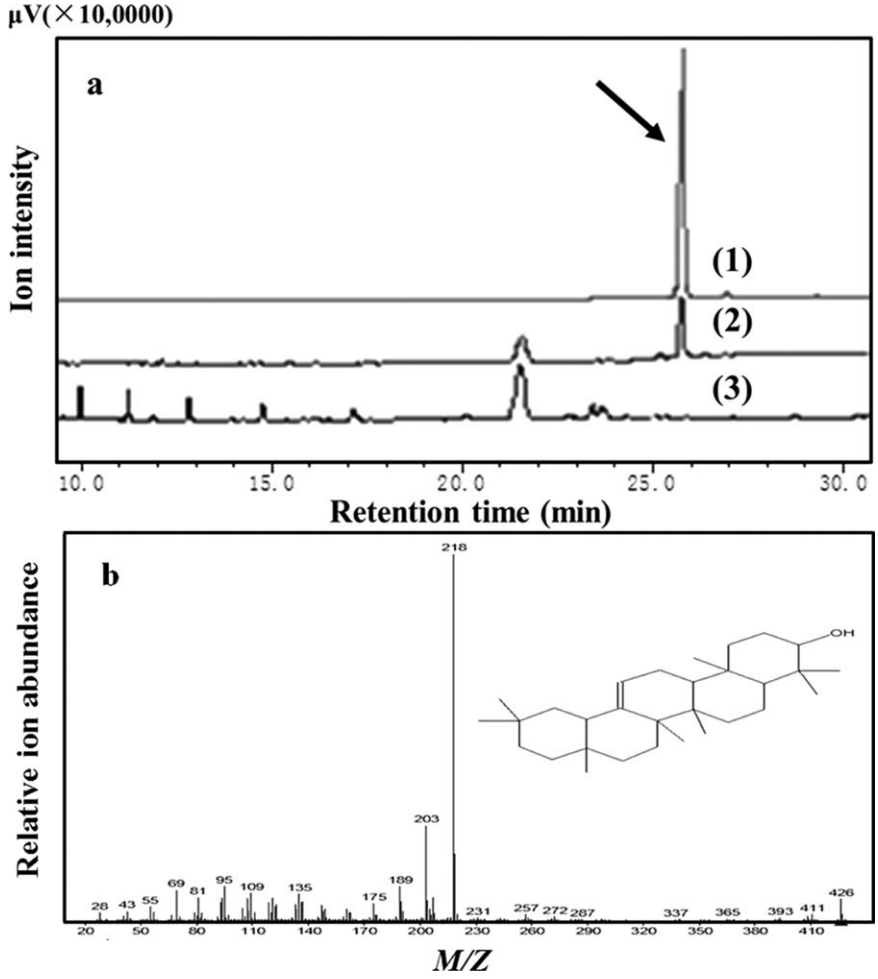

Figure 4. GC/MS analysis of β-amyrin produced by engineered *S. cerevisiae*.

(a) GC/MS chromatograms of standard trimethylsilyl β-amyrin (1), hexane extracts from SpKb cells expressing *bAS* (2) and from yeast cells transformed with the empty pRS42K vector (3). (b) MS spectrum of the peak denoted by arrow.

4.16 mg/L β-amyrin with a yield of 0.50 mg/g dry cell and productivity of 0.03 mg/(L·h) (Figure 5).

Dragging squalene flux to β-amyrin production by expressing a heterologous squalene monooxygenase

Squalene monooxygenase catalyzes squalene forming 2,3-oxidosqualene, the precursor both for β-amyrin and sterol synthesis. Because squalene monooxygenase exhibits very low specific activity in *S. cerevisiae*, this reaction is known as rate-limiting step in sterol biosynthetic pathway.[20] Previous work has explicitly described that 2,3-oxidosqualene accumulation is not observed during the cell growth.[20,21] Overexpression of squalene monooxygenase will lead a high increase of lanosterol for later ergosterol synthesis pathway.[20,21] Keeping in view, impact of high activity squalene monooxygenase from *C. albicans*[22] on β-amyrin production was investigated and two plasmid-based strains SpHsb, SpKsb and one genome-integrated SGsb were constructed; and transcription was confirmed by qPCR. In strain SpKsb, the transcription level of *ERG1* was 1.8-folds higher than SpHsb; in SGsb, the mRNA level was about 1.2-folds higher compared to SpKsb but 36% lower than SpHsb (Figure 3). The strains SpKsb and SpHsb engineered via this strategy could produce 5.80 and 12.86 mg/L of β-amyrin, which increased by 7.7- and 3.1-folds compared with SpKb and SpHb respectively (Figure 5). The

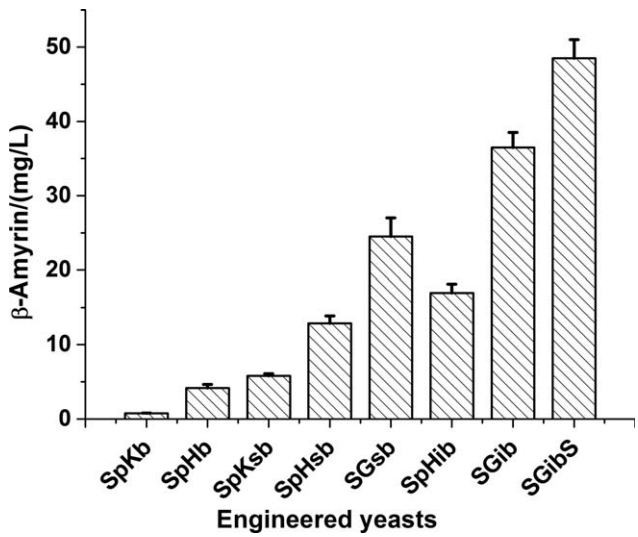

Figure 5. β-amyrin production with batch cultivation by engineered yeasts.

β-Amyrin was quantified at 120 h with a medium containing 2% glucose. Data are the means ± standard deviations of independent triplicates.

production level in SGsb reached 24.50 mg/L with a yield of 2.85 mg/g dry cell, increased about 33- and 40-folds compared to SpKb, respectively (Figure 5). The productivity reached 0.20 mg/(L·h), which was 33-folds of that produced by strain SpKb. These results showed that overexpressing a heterologous squalene monooxygenase efficiently dragged squalene to 2,3-oxidosqualene for β-amyrin synthesis.

Further enhancing β-amyrin production by enriching the pool of squalene

Numerous reports have demonstrated that the supply of FPP and squalene are major bottlenecks for terpenoid overproduction.[12,23] Comprehensive analysis of their concentrations in SpHsb and SGsb indicated a low level of these metabolites (Figure 7b). With a purpose to enrich the pool of squalene, *IDI* from *E. coli*, *ERG20* and *ERG9* from *S. cerevisiae* were expressed in SpHsb and SGsb, hence generated the plasmid-based SpHib and genome-integrated SGib strains (results of construction and colony PCR are shown in Figure S2 and S3, Supporting Information). Transcription level of *ERG20* and *ERG9* sufficiently increased 3.3- and 2.8-folds in SpHib and SGib, respectively, compared to wild yeast (Figure 3). Batch fermentation showed that coexpression of these genes had a positive effect on β-amyrin production, that is, a 31% increase in SpHib compared to SpHsb. Moreover, strain SGib greatly improved β-amyrin production, up to 36.50 mg/L with a yield of 4.56 mg/g dry cell and productivity of 0.30 mg/(L·d). This titer was almost 49-folds increase compared to SpKb (Figure 5). Such results verified the notion that β-amyrin production could be boosted via balancing and enhancing the supply of squalene.

Increasing β-amyrin titer by transcriptionally regulating metabolic flux

Transcriptional activator UPC2-1, a semidominant mutant allele of UPC2, was previously overexpressed to regulate the terpenoid production in *S. cerevisiae*, but only a modest effect was detected.[24,25] Investigations show that UPC2 upregulates sterol biosynthesis pathway by specifically binding to the promoters of this pathway. Analysis of promoters in sterol biosynthesis of *S. cerevisiae* discovered the putative sterol regulatory element (SRE) in the most promoters (Figure S4, Supporting Information). Presumptively, reconstructing of SRE into other promoters would recruit UPC2 and upregulate the expression of target gene. Therefore, the hired promoters were engineered by implanting a 50-bp sequence including 7-bp **TCGTATA** responsible for binding of UPC2. Using eGFP expression to validate these promoter strengths, it was found that the promoter strengths were significantly improved by 42.2–67.5% (Figure 6). Reconstructing of SRE made the strength of P_{ADH1} equivalent to P_{TEF1} regarded as the strongest promoter in yeast or even exceed the strength of wild P_{TEF1}, such as P_{ALA1}, P_{GPM1} and P_{FBA1}.[26]

The strain SGibS was thus refactored using the reconstructed promoters with SRE, which enhanced transcription level of target genes by 1.2–1.6-folds compared with SGib, demonstrating the obvious increase of the whole expression level of β-amyrin biosynthetic pathway (Figure 3). Such manipulation did not change the growth of yeast cell and ergosterol accumulation (Figures 7a, c). As expected, β-amyrin titer and yield was increased by about 35 and 29%, respectively, along with the 4-folds increase of squalene accumulation (Figures 5 and 7c). These results indicate that the strategy to engineer the binding site of UPC2 takes effect in regulating the transcription of target pathway. Through stepwise engineering and directed flux regulation, β-amyrin production potential of SGibS was lifted up about 65-fold vs. SpKb background. For increasing the production of target triterpenoid, the directional metabolic flux at transcriptional level by artificial construction of binding site for UPC2 was thus availably.

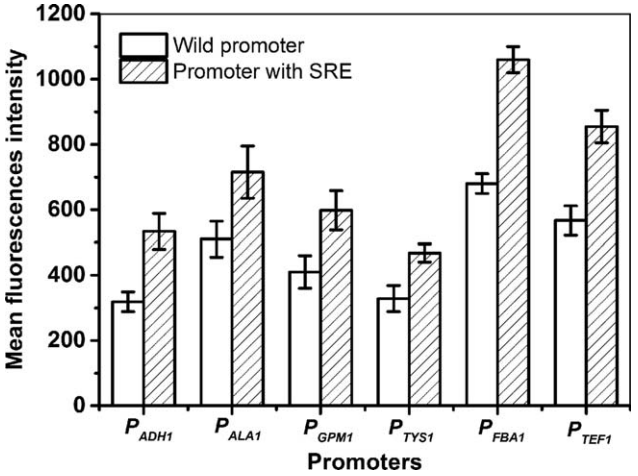

Figure 6. Verification of the promoter strength using eGFP as reporter by flow cytometry.

β-amyrin synthesis in fed-batch fermentation

The strains SGib and SGibS were selected for β-amyrin production in 5-L fermentor. After 24-h fermentation, 5 g/L glucose or ethanol was supplemented every 12 h. Figure 8 demonstrates the final titer for β-amyrin production in yeast strains. The similar ergosterol accumulation in SGib and SGibS was observed in both fed-batch and shake flask fermentations, further demonstrating that transcriptional regulation through refactored promoters using SRE only directed flux to β-amyrin production instead of sterol pathway. β-amyrin titer in both strains was increased sequentially in the whole fermentation. In glucose fed-batch fermentation mode, SGibS produced β-amyrin of 108.60 mg/L with a yield of 12.07 mg/g dry cell and productivity of 0.75 mg/(L·h), higher than SGib where was 75.50 mg/L, with yield of 8.39 mg/g dry cell and productivity of 0.530 mg/(L·h) (Figure 8a). Using ethanol feed mode, SGibS produced the highest β-amyrin of 138.80 mg/L with a yield of 16.30 mg/g dry cell and productivity of 0.96 mg/(L·h), almost 185-, 232-, and 154-folds higher than the initially engineered strain SpKb. Additional, 31.70 mg/L squalene in SGibS about 6.5-folds higher than that of SGib was also achieved (Figure 8b).

Discussion

The use of yeast platform for terpenoid production is promising to provide a more consistent and uniform product supply than the direct extraction from natural sources.[27] Previous studies have reported successful examples for expressing plant-derived terpenoid synthases in *S. cerevisiae*.[23–25] Except triterpenoid, several mono-, sesqui-, di-, and tetraterpenoids with high-titer such as linalool, amorphadiene, taxol, carotenoids had been produced in engineered *S. cerevisiae*.[12,25,28,29] In this work, *S. cerevisiae* was engineered to produce triterpenoid β-amyrin. As observed in Figure 4, β-amyrin was successfully synthesized by β-amyrin synthase from *G. glabra*. However, more β-amyrin was produced when β-amyrin

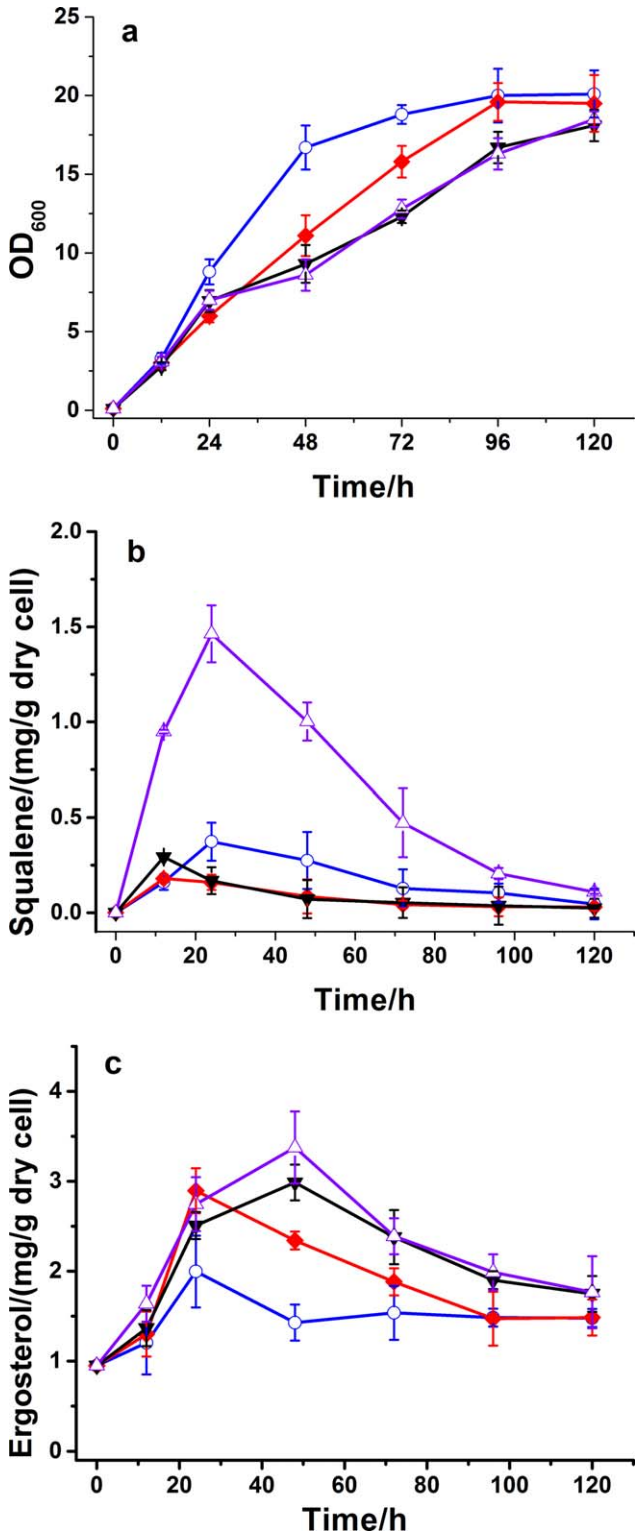

Figure 7. Cell growth and metabolite levels of various engineered yeasts in batch cultivation.

(a) Growth of various engineered *S. cerevisiae*. (b) Squalene accumulation in various engineered *S. cerevisiae*. (c) Ergosterol production in various engineered *S. cerevisiae*. Engineered *S.cerevisiae* SpHsb (○), SGsb (◆), SGib (▼), and SGibS (△). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

synthase was expressed using single copy plasmid (SpHb, SpHsb) than using multi-copy plasmid (SpKb, SpKsb). This effect could be caused by pathway stability issues or metabolic burden to maintain plasmids with different copy numbers and antibiotic resistance genes. Most likely, issues such as plasmid segregation and imprecise control of plasmid-based gene expression are amplified with the copy number increase of plasmid.[30] The detailed and deep mechanisms need further investigate though the similar phenomenon was also observed in the other report.[31]

Most studies aiming at enhancing terpenoid production have used overexpression of key flux-regulating enzymes.[12,25,31] 2,3-Oxidosqualene is the common starting point for cyclization reactions in both triterpenoid and sterol biosynthesis, and formed by epoxidation of the double bond of squalene via squalene monooxygenase, a pivotal enzyme for triterpenoid biosynthesis.[16,32] Upregulation of squalene monooxygenase previously resulted in enhanced sterol accumulation.[33] In this work, expressing a squalene monooxygenase derived from *C. albicans* increased β-amyrin production 2.7- to 5.1-folds, suggesting that construction of a powerful heterologous supply line for 2,3-oxidosqualene would be imperative to drag the squalene flux for increasing the triterpenoid production. FPP subsists at branch point of terpenoids pathway and its intracellular concentration is tightly regulated only coupled with the cellular demand to produce sterol during normal growth conditions.[34] FPP-derived squalene is another critical precursor both for triterpenoids and number of other essential compounds including sterol.[35] Expression of squalene synthase would enhance β-amyrin synthesis.[36] To replenish the pool of squalene, FPP, and squalene synthase along with IPP isomerase were thus overexpressed and improved β-amyrin production by 23–28%, indicating that upregulation of these genes enhanced the availability of squalene and diverted the flow to β-amyrin. However, we observed that enriching the squalene pool did not cause the increase of squalene-dependent ergosterol (Figures 7b and 8). Redundant squalene would be stored in cellular membranes especially in microsomes as an unconventional example of nonpolar lipid storage,[37] or be restricted after postsqualene part of ergosterol pathway such as precursor for heme production and control of intermediates flux to end-products.[37,38]

Although UPC2-1 has been overexpressed to augment terpenoid production, the extensive regulation of endogenous pathways at both the transcriptional and posttranscriptional level in *S. cerevisiae* limit its function and even lead to a negative effect.[25,39] Based on the analysis of UPC2 regulation metabolism, its binding site was for the first time engineered into promoters in engineered pathway for β-amyrin synthesis. The present work successfully upregulated the transcriptional level of engineered pathway and increased β-amyrin production by 35%. Such engineering strategy has well achieved the similar positive effect on terpenoid production as combinational expression of truncated 3-hydroxyl-3-methylglutaryl-CoA reductase and UPC2-1.[25] However, further overexpressing UPC2-1 in the strains of SGib and SGibS (defined as SGibU and SGibSU strains, respectively) caused more than 2.5-folds increase of squalene accumulation and a greater increase of ergosterol (Supporting Information methods 1.3 and Table S6). Although overexpressing UPC2-1 in SGibS strain increased more β-amyrin (3.8%) than that in SGib (2.5%), the positive effect was slight, demonstrating that regulating terpenoid pathway through refactored promoters with SRE is more effective than that of overexpressing UPC2-1

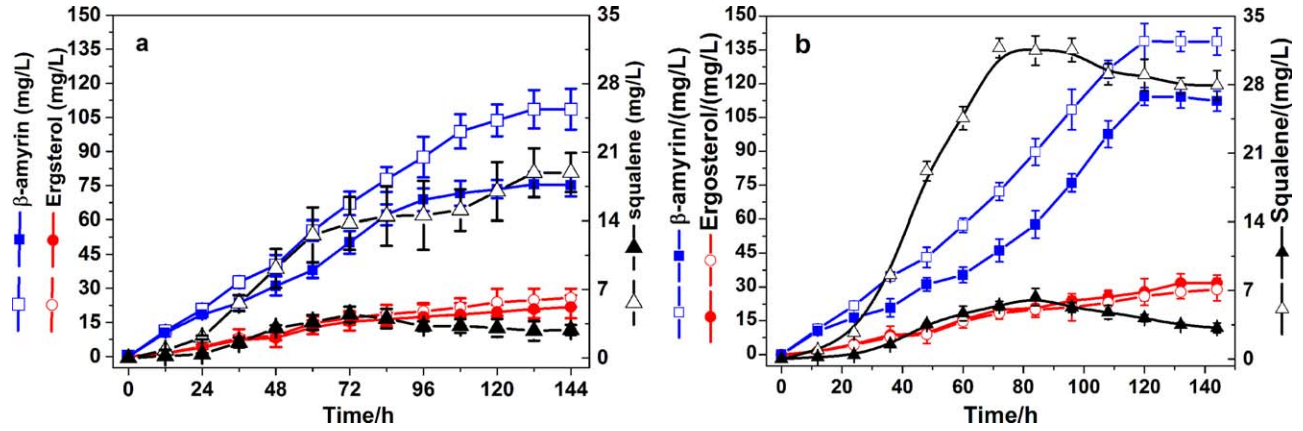

Figure 8. Time course of β-amyrin production in fed-batch cultivation.

β-Amyrin production, squalene and ergosterol accumulation in SGib (■,▲, and •) and SGibS (□, △, and ○) were compared in glucose (a) and ethanol (b) fed-batch fermentations. Samples from three biological replicates were taken every 12 h for a 144 h period for products quantification by GC. The error bars indicate standard deviation.[Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

(Table S6, Supporting Information). The reconstructed promoters with SRE including P_{ALA1}, P_{GPM1}, and P_{FBA1} were used to replace corresponding wild promoters in xylitol producing *S. cerevisiae* that was previously constructed by our group,[40] and xylitol production was improved 29.8% (Supporting Information methods 1.4 and Figure S5), showing the expandability of this strategy. Therefore, the strategy using fused UPC2 binding site to regulate pathway is not exclusive to β-amyrin, and may serve as a novel general road for balancing endogenous and exogenous pathways of engineered yeast.

In addition, compared to plasmid-based strains, all genome-integrated strains were observed having better β-amyrin producing capacity. Experiments to measure the plasmid stability of SpHb, SpHsb, and SpHib indicated that plasmids in three strains exhibited a decrease over 48-h cultivation. The plasmid loss increased with the increase of gene number assembled in plasmid, of three strains the loss of plasmid in SpHib was close to 30% at 120-h cultivation (Supporting Information methods 1.5 and Figure S6). Supposedly, the increase of exogenous gene number in a plasmid would increase the burden of plasmid, resulting in physiological change of cells and finally leading to plasmid loss. Alternatively, genome integration not only can steadily express genes instead of decreasing the number of genes during the whole fermentation, but also can avoids drawbacks such as segregation and plasmid maintenance-associated metabolic stress.[30] It is apparent that expressing multigene using genome-integrated way is a good strategy for pathway engineering. Moreover, techniques for DNA assembly can rapidly one-step assemble large biosynthetic pathways into genome. In this study, DNA assembler relying on the native cellular homologous recombination machinery of *S. cerevisiae* efficiently assemble β-amyrin pathway with five-gene cassettes. Using this method, gene cassettes were directly assembled in a prescribed order through terminal overlap sequences of DNA fragments, showing its potential in the field of combinatorial biosynthesis.[17] Finally, with the help of ethanol fed-batch fermentation the β-amyrin titer and yield reached 138.80 mg/L and 16.30 mg/g dry cell, respectively. To our best knowledge, 6 mg/L β-amyrin was the best reported yield produced by an engineered *S. cerevisiae*, which was obtained through the expression of β-amyrin synthase from *Artemisia annua* with the help of downregulation lanosterol synthase and overexpression of tHMG1.[16] Compared with the extraction procedure from plants,[10] microbial process with the high titer and yield developed in this study is expected to reduce the cost of β-amyrin production, showing a great potential for the industrial application.

Conclusions

A strategy combining stepwise metabolic engineering and flux control at transcriptional level in *S. cerevisiae* was developed to enhance triterpenoid production. As a result, an efficient biosynthetic pathway for producing β-amyrin in yeast was constructed. Expressing a heterologous squalene monooxygenase efficiently dragged the precursor squalene to β-amyrin. Increasing squalene pool greatly enhanced β-amyrin production. Engineering SRE responsible for binding UPC2 into the hired promoters achieved redirection of metabolic flux at transcriptional level, resulting in a 65-fold increase of β-amyrin production. With the help of ethanol fed-batch fermentation, β-amyrin titer was improved up to 138.80 mg/L with a yield of 16.30 mg/g dry cell which were best in present reports. The developed microbial process is thus expected to reduce the extraction cost of β-amyrin from plants. The research results also demonstrated that metabolic engineering strategy based on transcription activation maybe open a novel road for intentionally balancing the endogenous and exogenous pathways in engineered yeast.

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