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Combinatorial Assembly of Large Biochemical Pathways into Yeast Chromosomes for Improved Production of Value-added Compounds

Jifeng Yuan*[,†,‡] and Chi Bun Ching[†,‡,§]

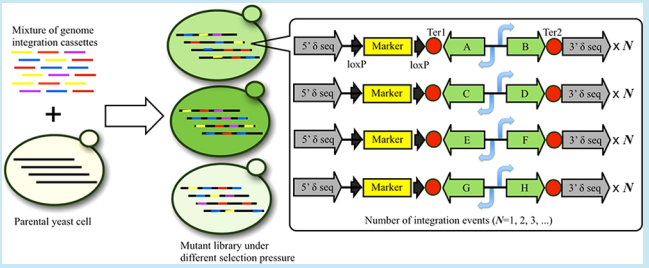
[†]Department of Chemical and Biomolecular Engineering, National University of Singapore, 4 Engineering Drive 4, Singapore 117585, Singapore

[‡]Synthetic Biology Research Consortium, National University of Singapore, 28 Medical Drive, Singapore 117456, Singapore

[§]Singapore Institute of Technology, 10 Dover Drive, Singapore 138683, Singapore

Supporting Information

ABSTRACT: *Saccharomyces cerevisiae* as a eukaryotic organism is particularly suitable as microbial cell factory because it has interesting features such as membrane environments for supporting membrane-associated enzymes and its capability for post-translational modifications of enzymes from plants. However, *S. cerevisiae* does not readily express polycistronic transcriptional units, which represents a significant challenge for constructing large biochemical pathways in budding yeast. In the present study, we developed a novel approach for rapid construction of large biochemical pathways into yeast chromosomes. Our approach takes advantage of antibiotic selection for combinatorial assembly of large pathways into the δ-sites of retrotransposon elements of yeast chromosomes. As proof-of-principle, a five-gene isobutanol pathway and an eight-gene mevalonate pathway were successfully assembled into yeast chromosomes in one-step fashion. To our knowledge, this is the first report to exploit δ-integration coupled with antibiotic selection for rapid assembly of large biochemical pathways in budding yeast. We envision our new approach could serve as a generalized technique for large pathway construction in yeast—a method that would be of significant interest to the synthetic biology community.



KEYWORDS: *mevalonate pathway, isobutanol pathway, pathway assembly, δ-integration, antibiotic selection, Saccharomyces cerevisiae*

Among microorganisms commonly exploited as microbial cell factories, *Saccharomyces cerevisiae* is particularly suitable to host diverse metabolite biosynthetic pathways. As an industrial microorganism commonly used in food and beverage production, *S. cerevisiae* is also a key model organism for fundamental molecular biology research, and it is the first eukaryotic organism with its genome completely sequenced.[1] Based on the knowledge obtained throughout these years, various tools for pathway construction have been developed in *S. cerevisiae*, such as methods for controlling expression levels of heterologous genes, rapidly assembling of large biochemical pathways, and characterizing different chromosomal sites for heterologous gene expressions.[2−5] Moreover, *S. cerevisiae* as a eukaryotic organism has the environment provided by an endomembrane such as the endoplasmic reticulum or mitochondrial inner membrane, for supporting the functional expression of membrane associated enzymes such as cytochrome P450s.[6,7] Another interesting feature of exploiting *S. cerevisiae* as microbial cell factory is its capability for post-translational modifications of enzymes from plants and mammals.[8]

Despite all these advantages listed here, *S. cerevisiae* does not readily express multigene (polycistronic) transcriptional units to allow the coordinated expression of many genes within a compact operon. Thus, it will be of great interest to develop new methods for rapid design and construction of large biochemical pathways in *S. cerevisiae*, especially for synthetic biology and metabolic engineering applications. Recently, Huimin Zhao and co-workers developed a new method, called "DNA assembler", that enables rapid construction of large biochemical pathways in one-step fashion in *S. cerevisiae*.[4,9] This method harnesses the high efficiency of recombination system of budding yeast, which has been widely used for plasmid construction, library creation, and even for bacterial genome assembly.[10−12] With overlapping homology more than 40 bp between individual cassettes, *S. cerevisiae* is capable of assembling a xylose utilization pathway and a zeaxanthin biosynthesis pathway into a vector or integrated into the chromosome.

In a separate study, cocktail δ-integration coupled with different auxotrophic selection markers has been developed for constructing ratio-optimized cellulolytic enzyme expression in yeast.[13] Here, we sought to investigate δ-integration coupled

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with antibiotic selection for rapid construction of large biochemical pathways into yeast chromosomes. Specifically, the new δ-integration platform was modified with bidirectional promoter system to enable rapid assembly of large biochemical pathways in one-step fashion at δ-sites of retrotransposons (Ty) elements[14,15] (Figure 1). As proof-of-concept, we first

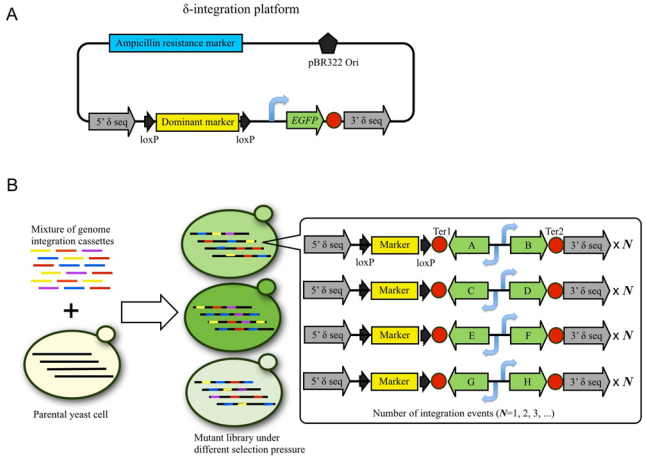

Figure 1. Schematic diagram of δ-integration platform for the rapid construction of large biochemical pathways into yeast chromosomes. (A) Plasmid template for δ-integration comprises homologous sequences to δ-site of Ty elements and antibiotic selection marker to facilitate multiple integrations through modulating the selection pressure of antibiotics. Sequence from 5′-δ to 3′-δ will be amplified by PCR and used as genome integration cassette. (B) Experimental procedure of new δ-integration approach for assembling multiple-gene pathway into yeast chromosomes. To integrate the entire biochemical pathway into yeast chromosomes, high concentration of antibiotics is used so that *N* for individual cassette is >1. MCS stands for multiple cloning sites. Ter stands for terminator.

exploited the new δ-integration platform to rapidly assemble a mitochondrion-based isobutanol pathway in budding yeast for high-level production of isobutanol[16] (Supporting Information (SI) Figure S1). We found the entire isobutanol pathway was successfully assembled into yeast chromosomes when high antibiotic selection was applied. The best variant yielded approximately 600 mg/L of isobutanol after 4 days cultivation, which was among the highest reported titers in budding yeast. Next, we sought to assemble an eight-gene mevalonate pathway into yeast chromosomes for overexpression, with the aim of engineering yeast strains to be served as platform microbe for high-level production of terpenoid derivatives that have great commercial values to serve as therapeutics.[6,17−19] In particular, engineered strains were tested for amorpha-4,11-diene production, an important precursor to artimisinin—a highly valuable antimalarial pharmaceutical (SI Figure S2).[6] All engineered strains showed substantial improvement of amorpha-4,11-diene levels, to a titer around 100 mg/L. Among them, the best variant produced 120 mg/L of amorpha-4,11-diene after 5 days cultivation. To further improve the amorpha-4,11-diene titer, engineered strains were modified by restricting the metabolic flux toward ergosterol biosynthesis and adjusting the expression level of amorpha-4,11-diene synthase. Small-scale studies revealed that engineered strain with centromeric expression of amorpha-4,11-diene synthase was capable of producing approximately 500 mg/L of amorpha-4,11-diene when *ERG9* expression was restricted, which represented the best titer of amorpha-4,11-diene in the laboratory strain of S288C.

RESULTS AND DISCUSSION

Combinatorial Assembly of Mitochondrion-Based Isobutanol Pathway into Yeast Chromosomes. For synthetic biology applications, it often requires the transfer of the entire biosynthetic pathways from native hosts such as plants and mammals into microbes to achieve high-level production of value-added chemicals through large-scale fermentation processes. Thus, we decided to investigate whether it is possible to harness the δ-sites of retrotransposons (Ty) elements[14,15] coupled with antibiotic selection for the rapid construction of large biochemical pathways into yeast chromosomes (Figure 1). The idea was originated from the approach called chemically induced chromosomal evolution (CIChE), which has been used to evolve *E. coli* chromosome for gene duplications using antibiotic of chloramphenicol.[20] In the present study, we chose to use dominant antibiotic selection marker against phleomycin (a glycopeptide antibiotic of the bleomycin family that can introduce DNA breaks), as very low concentration of phleomycin (10 μg/mL) is sufficient for the selection of gene deletion events.[21]

Before exploiting combinatorial integration of biochemical pathways into yeast chromosomes, the δ-integration platform was first tested for integrating a reporter gene encoding enhanced green fluorescent protein (EGFP) to examine whether it is possible to achieve various degrees of integration events by simply modulating the concentration of antibiotics. As can be seen in Figure 2, engineered strains obtained from

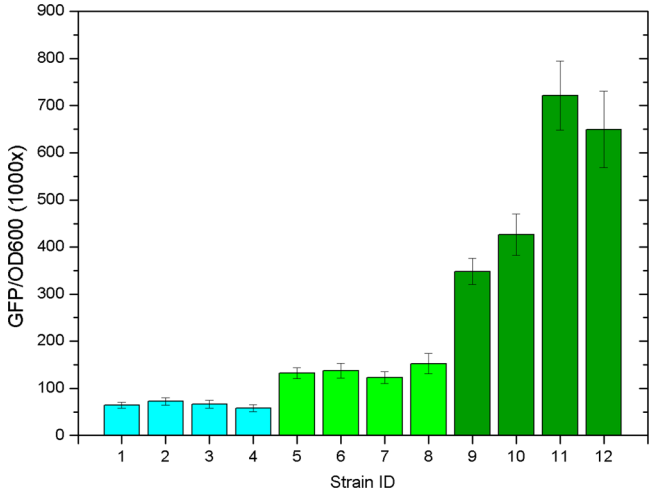

Figure 2. Green fluorescence intensities for engineered strains obtained from library with cells adapted to various concentrations of antibiotics. Strains 1−4 were obtained from library treated with 40 μg/mL of phleomycin. Strains 5−8 and strains 9−12 were from library supplemented with 80 and 160 μg/mL of phleomycin, respectively. Green fluorescence intensities were recorded with excitation/emission at 476/512 nm. Experiments were performed in triplicate.

library supplemented with higher concentration of antibiotics showed significantly higher levels of fluorescent intensities over engineered strains from library with lower selection pressure, which confirmed that the feasibility of modulating genome integration events by simply selecting with different concentrations of antibiotics.

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Previously, the isobutanol pathway has been constructed in their natural compartments by overexpressing only some of pathway genes to increase isobutanol production.[22−24] Simple overexpression of the isobutanol pathway genes in their natural compartments to improve isobutanol titer is not only limited by the transport of intermediates from mitochondrion to cytoplasm but also limited by competing pathways. In contrast, eliminating bottleneck posed by the transport of intermediates across membranes showed great promise for high-level production of isobutanol, to a titer around 650 mg/L by either relocalization of the upstream isobutanol biosynthetic pathway to cytoplasm[25] or sequestration of downstream pathway into mitochondria.[16] In the present study, we aimed to further optimize mitochondrion-based isobutanol biosynthetic pathway by eliminating plasmid-based overexpression system and combinatorially balancing of pathway gene expression levels. To achieve mitochondrion-based isobutanol production, α-ketoacid decarboxylase (encoded by *ARO10*) and alcohol dehydrogenase (encoded by *ADH7*) were fused with the N-terminal mitochondrial localization signal from subunit IV of the yeast cytochrome c oxidase (encoded by *COX4*),[26] in a similar way as carried out by previous study.[16] Here, we proceeded to construct isobutanol-producing mutant libraries, and we sought to use three different concentrations of antibiotics (40 μg/mL, 80 μg/mL, and 160 μg/mL of phleomycin) for different degrees of genome integration events. To examine whether isobutanol pathway genes have been successfully integrated into yeast chromosomes, universal primer pairs F_GAL10Scr/R_GAL10Scr and F_GAL1Scr/R_GAL1Scr were used to verify genome integration events. As can be seen in SI Table S4, PCR results confirmed there were successful genome integration events for isobutanol pathway genes. Additionally, there was a clear trend of more isobutanol pathway genes being detected for engineered strains from library treated with high concentration of antibiotics. Interestingly, when the genome integration cassettes were partitioned into upstream and downstream modules, there was biased genome integration as genes with smaller size such as *ILV5* and *ADH7* (gene size around 1.2 kb) were integrated into yeast chromosomes for all engineered strains (SI Table S4). In contrast, the chance for *ILV2*, *ILV3*, and *ARO10* with size around 2 kb to be integrated into yeast chromosomes was lower. The entire isobutanol biosynthesis pathway was integrated into yeast chromosomes for the majority of engineered strains from library treated with 160 μg/mL of phleomycin. Based on these findings, biased integration should be considered for the future library construction and one possible solution would be adjusting DNA input in the mixture during electroporation.

As majority of engineered strains from the library supplemented with 160 μg/mL of phleomycin appeared to have the entire mitochondrion-based isobutanol pathway, we thus randomly tested 24 engineered strains from this library to determine whether these engineered strains could yield high levels of isobutanol. As can be seen from Figure 3A, distinct levels of isobutanol were observed for engineered strains after 4 days cultivation. There were trace amounts of C5 alcohols such as isopentanol and 2-methyl-1-butanol detected during GC-MS analysis (Figure 3B). The variations of isobutanol titers in engineered strains suggested that there should be beneficial combinations of integration events for higher-level production of isobutanol. It was surprising to find out that poor cell densities did not correlate with isobutanol levels (SI Figure S3).

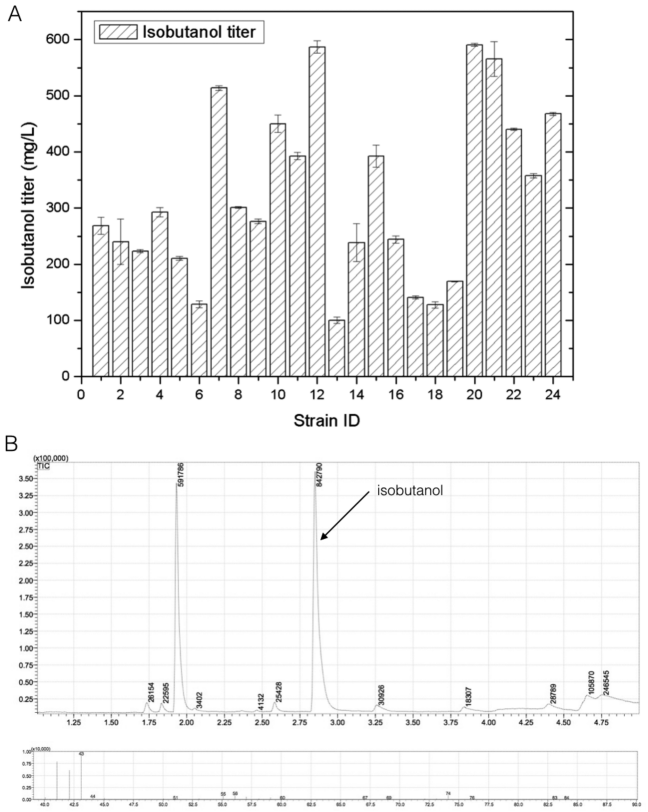

Figure 3. Characterization of isobutanol productions in engineered *S. cerevisiae* BY4742 strains. (A) Isobutanol levels in engineered strains under shake-tube condition. (B) Representative GC-MS result for the best producer M20. Twenty-four engineered strains from library supplemented with high concentration of antibiotics (160 μg/mL of phleomycin) were subjected to characterization. Alcohol levels were measured after 4 days cultivation using GC-MS. For the quantitation of isobutanol produced by engineered yeast cells, authentic alcohol compound was used for plotting standard curve. Data represented the average and standard deviation of three independent experiments.

For example, engineered strain M8 and M16 with poor growth did not produce the highest levels of isobutanol; however, the best producers such as M12 and M20 showed only minor growth impairment when compared to the parental strain without genetic modification (data not shown). However, there was good correlation of ethanol levels with cell densities as shown from SI Figure S3. Since no detectable accumulation of intermediates such as isobutanal was observed during GC-MS analysis for all engineered strains, it suggested that the enzymatic activity of alcohol dehydrogenase encoded by *ADH7* was sufficient to convert isobutanal into isobutanol.

Among 24 engineered strains, the best variant (M20) produced approximately 600 mg/L of isobutanol (Figure 3A), which was impressive as only one round of combinatorial genome integration was performed. In comparison, the reference strain without genetic modification only produced approximately 6 mg/L of isobutanol (SI Figure S4). Here, the resulted strains did produce isobutanol more efficiently than plasmid-based expression system as significantly less sugar was consumed,[16] which confirmed that eliminating plasmid burden and combinatorially balancing metabolic flux could generate yeast strains with better performance. However, as isobutanol was reported to reach 20 g/L in the engineered *E. coli*,[27−29] there is still a significant room for improvement in yeast to

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achieve industrial-scale production of isobutanol to be considered as gasoline substitutes.

To confirm the improvement of isobutanol titer in the engineered strains was attributed to the overexpression of isobutanol pathway genes, we decided to verify of gene expression profile of M20 by carrying out quantitative real-time reverse-transcription PCR (qRT-PCR) studies. To correct for differences in the amounts of starting materials, *ACT1* was chosen as a reference housekeeping gene and the results were presented as ratios of gene expression between the target gene (gene of interest) and the reference gene, *ACT1*.[30] As can be seen from Figure 4, gene expression levels of all isobutanol

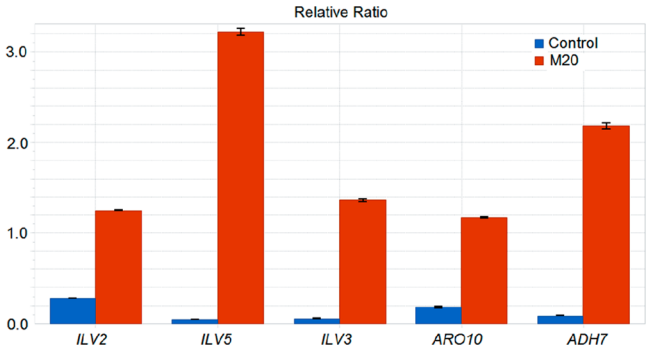

Figure 4. qRT-PCR studies to determine overexpression of isobutanol pathway genes. The engineered strain M20 and the parental strain *S. cerevisiae* BY4742 were inoculated in SC media at initial OD_{600} of 0.05. After 12 h of cultivation, RNA was extracted from cell culture and further converted to cDNA. qRT-PCR experiments were performed to determine the relative abundances of isobutanol pathway genes in each strain with respect to that of *ACT1*, which encodes actin and serves as an internal control. Experiments were carried out in triplicate.

pathway genes in M20 were significantly higher when compared to those of the parental strain, which confirmed all isobutanol pathway genes were successfully integrated into yeast chromosomes for overexpression. Among them, *ILV5* and *ADH7* showed the highest abundances of mRNA levels, presumably due to multiple copies of these genes into yeast chromosomes.

Combinatorial Assembly of the Mevalonate Pathway into Yeast Chromosomes. Upon successful assembly of isobutanol pathway, we further attempted to reconstruct the eight-gene mevalonate pathway into yeast chromosomes. Here, even higher concentration of antibiotics for selection (240 μg/mL of phleomycin) was used to ensure the entire mevalonate pathway to be integrated into yeast chromosomes. As expected, when eight randomly picked engineered strains were subjected to PCR verification, all engineered strains showed eight-band pattern during PCR verification, which confirmed all mevalonate pathway genes were successfully integrated into yeast chromosomes (SI Figure S5).

Next, we sought to exploit these engineered strains as platform microbes for high-level production of terpenoids, and all eight engineered strains as mentioned above were directly tested for the production of amorpha-4,11-diene, an important precursor for antimalarial drug artimisinin (SI Figure S2). As can be seen in Figure 5, all engineered strains produced high-levels of amorpha-4,11-diene, with titer around 100 mg/L in small-scale shake-tube condition. Among them, the best variant Y104 actually produced around 120 mg/L after 5 days cultivation. Surprisingly, the parental strain transformed with

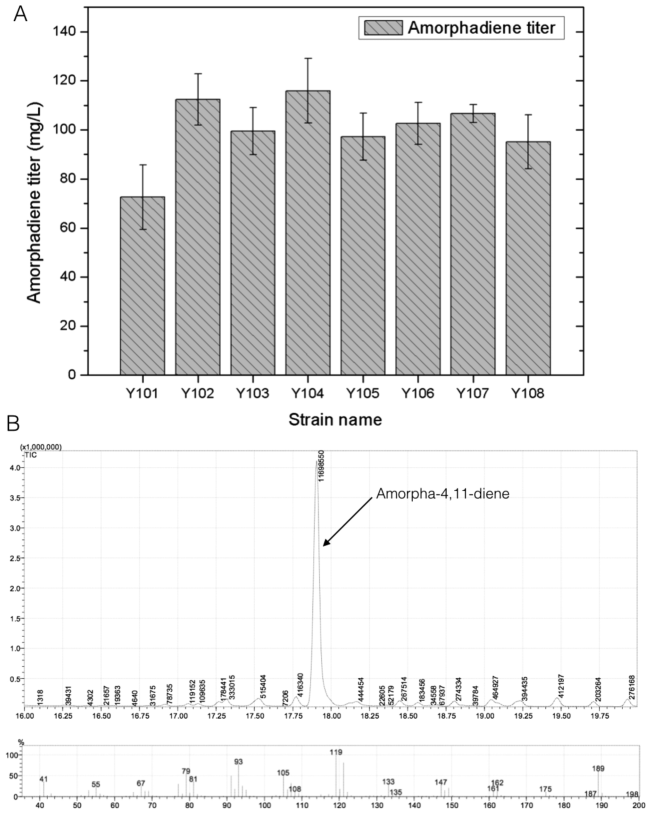

Figure 5. Characterization of amorpha-4,11-diene production for the engineered strains with the entire mevalonate pathway integrated into yeast chromosomes. (A) Amorpha-4,11-diene levels in engineered strains. (B) Representative GC-MS profile for the best producer Y104. Engineered yeast strains were transformed with plasmid pYES2ADS harboring the codon-optimized version of amorpha-4,11-diene synthase from *A. annua*. Engineered strains were cultured in SC medium with uracil dropped out. Amorpha-4,11-diene levels were measured after 5 days. Data represented the average and standard deviation of three independent experiments.

pYES2ADS only produced around 2 mg/L of amorpha-4,11-diene under the same experimental condition (SI Figure S4), which was lower compared to 4.4 mg/L for the same strain harboring pRS425ADS as reported previously.[6] Further qRT-PCR analysis of gene expression levels of Y104 confirmed that mRNA levels of all mevalonate pathway genes were overexpressed (as shown in Figure 6). Among them, *tHMG1* and *ERG10* showed the highest expression levels and mRNA abundances of both genes were approximately 7-fold over that of internal control *ACT1* (Figure 6). Interestingly, mRNA abundances for *ERG8* and *ERG19* were relatively low compared to other mevalonate pathway genes, which probably have beneficial effect on isoprenoid productions as previous investigation showed overexpression of *ERG19* would lead to reduced sterol content and the accumulation of diphosphate intermediates led to feedback inhibitions.[31] We also found all the genes put under control of pGAL1 promoter (*ERG10*, *tHMG1*, *ERG8*, and *ERG20*) showed slightly higher mRNA abundance over those put under control of pGAL10 promoter (*ERG13*, *ERG12*, *ERG19*, and *IDI1*), which further supported that pGAL1 promoter is generally considered to be stronger over pGAL10 as previously reported.[32,33] Moreover, the possibility of cross talk between different cassettes appears to be negligible, as relative levels for genes in the same cassette

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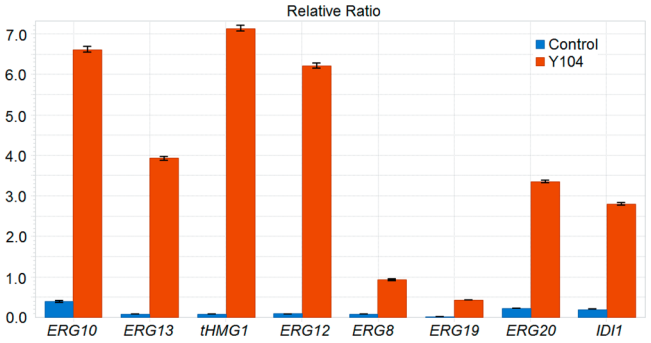

Figure 6. qRT-PCR analysis of the engineered strain Y104 to determine the expression levels of mevalonate pathway genes. Two cultures of each strain, the parental strain *S. cerevisiae* BY4742 and the engineered strain Y104, were inoculated in SC media at initial OD_{600} of 0.05 and harvested at exponential phase. Data represented the relative abundances of mevalonate pathway genes in each strain with respect to that of *ACT1*. Experiment was carried out in triplicate.

were almost consistent. For example, mRNA abundance of *tHMG1* under control of pGAL1 over that of *ERG12* under control of pGAL10 is similar to that of *ERG8* over *ERG19* (Figure 6).

Based on previous findings,[6,34] high-level production of non-native isoprenoid products requires that FPP flux be diverted from the production of sterols to the heterologous metabolic reactions. Down-regulation of *ERG9*, which encodes squalene synthase (the first committed step after FPP in sterol biosynthesis) by replacing its native promoter with the methionine-repressible MET3 promoter increased amorpha-4,11-diene production an additional 2-fold.[6] More recently, copper-regulated CTR3 promoter was used to restrict *ERG9* expression by addition of the inexpensive repressor copper sulfate to the medium, and CTR3 promoter for restricting *ERG9* expression showed similar effect on improving amorpha-4,11-diene titers.[35] Since the native CTR3 promoter in laboratory strain S288C is interrupted by the insertion of a Ty2 element,[36] we thus decided to use copper-regulated CTR3 promoter from *S. cerevisiae* CEN.PK2 strain instead, for restricting *ERG9* expression in Y104. The resulted strain with *ERG9* under control of copper-repressible promoter pCTR3 was designated as Y104A. To further examine whether modulating *ADS* expression levels would have profound effect on amorpha-4,11-diene productions, we constructed another centromeric plasmid to express *ADS* gene (pRS416ADS). We found strain Y104A harboring pYES2ADS produced approximately 250 mg/L amorpha-4,11-diene under *ERG9* restriction, which confirmed the copper-regulated CTR3 promoter did repress the metabolic flux toward ergosterol biosynthesis and divert the flux to enhanced levels of amporpha-4,11-diene. Interestingly, strain Y104A with pRS416ADS yielded even higher level of amorpha-4,11-diene, to a titer around 500 mg/L under the same condition, which represents the highest reported titer of amorpha-4,11-diene in the laboratory strain S288C. However, when the entire mevalonate pathway was overexpressed in CEN.PK2 strain, it was reported to produce 1200 mg/L of amorpha-4,11-diene under shake-flask condition.[37] In the near future, we will further attempt to engineer CEN.PK2 strain to utilize glucose as carbon source for high-level production of terpenoids and characterize the product yield under fed-batch condition.

Here, we have successfully demonstrated δ-integration coupled with antibiotic selection for rapid construction of large biochemical pathways into yeast chromosomes. When compared to DNA assembler that has been widely used for the rapid construction of biochemical pathways,[9,38] our approach offers several advantages. For example, DNA assembler cannot be used to adjust gene copy numbers for modulating enzyme expression levels, whereas multiple copies of genes involved in the rate limiting steps are expected to further improve the pathway activity as demonstrated by previous studies.[6,37] Since yeast does not readily express polycistronic genes, different promoter and terminator flanking sequences for individual gene are required for the functional expression in yeast, and it is a must to avoid the reuse of identical or similar regulatory elements when assembling long biochemical pathway using DNA assembler method, as any internal recombination will result in incomplete biochemical pathways. In comparison, only one pair of well-characterized promoter and terminator was used for constructing the five-gene isobutanol pathway and the eight-gene mevalonate pathway using our current δ-integration platform, which eliminates the requirement of meticulously selected sets of appropriate promoter and terminator sequences prior to cassette assembly. Additionally, the feature of bidirectional promoter system adopted in the present study may facilitate promoter engineering for fine-tuning enzyme expression levels in a follow-up study to achieve further balanced metabolic flux and improved product titer, in a similar way to the procedure described by previous studies.[39,40] Moreover, when compared to cocktail δ-integration using different auxotrophic selection markers for constructing ratio-optimized cellulolytic enzyme expression in yeast,[13] our modified δ-integration with antibiotic selection marker can be easily used for modulating integration events to achieve ratio-optimized expression of pathway genes in one-step fashion, whereas auxotrophic selection marker will typically result in single integration for the cocktail δ-integration system and it will take several rounds of genetic manipulation to introduce more genes into yeast chromosomes. Noteworthy, for the concern of genetic stability of our constructed strains, it would be possible to stabilize the construct by simply deleting *Rad51* gene in the engineered strains, in a similar way to *RecA* deletion carried out by the previous study.[20]

Conclusions. In summary, we successfully demonstrated that our new δ-integration approach could effectively assemble large biochemical pathways into yeast chromosomes. To our knowledge, this is the first report to exploit δ-integration coupled with antibiotic selection to rapid assemble large biochemical pathways in budding yeast. Since this new method only requires simple plasmid preparation and one-step yeast transformation to assemble the entire biochemical pathway, our new approach represents a powerful tool in the construction of large biochemical pathways for synthetic biology, metabolic engineering, and pathway engineering studies.

METHODS

Strains, Plasmids, and Reagents. *Escherichia coli* strain DH5α or TOP10 were used for routine transformation and strains were cultivated at 37 °C in Luria−Bertani (LB) medium. *S. cerevisiae* BY4742 strain (MATα; *his3*Δ*1*; *leu2*Δ*0*; *lys2*Δ*0*; *ura3*Δ*0*) and CEN.PK2-1C were obtained from EUROSCARF. *S. cerevisiae* BY4742 strain was used as the parent strain for all yeast strain constructions. YPD medium was used for culturing parental strains and engineered strains without plasmid.

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Synthetic complete (SC) media with leucine and/or uracil dropped out where appropriate were used for maintaining engineered strains transformed with plasmids harboring different auxotrophic selection markers. For the induction of genes under the control of galactose inducible promoters, *S. cerevisiae* strains were grown in galactose as carbon source. Plasmid pUG66, pKT127, pSH47, and pUG73 were all obtained from EUROSCARF. Plasmid pUC18 and pYES2 were obtained from Invitrogen, Singapore. pESC-URA was purchased from Life Technologies, Singapore. Plasmid pRS425ADS harboring the codon optimized amorpha-4,11-diene synthase gene from *Artemisia annua*[6] was a gift from Prof. Jay Keasling at University of California, Berkeley. All the chemicals used in the present study were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Restriction enzymes, Taq polymerase, alkaline phosphatase (CIP), and T4 ligase were obtained from New England Biolabs (Beverly, MA, U.S.A.). iProof HF polymerase and iScript Reverse Transcription Supermix were purchased from BioRad (Hercules, CA, U.S.A.). QIAquick Gel Extraction Kit, QIAprep Spin Miniprep Kit, and RNeasy Mini Kit were all purchased from QIAGEN (Singapore). FastStart Essential DNA Green Master was purchased from Roche. Oligonucleotides were synthesized by integrated DNA technologies (Singapore). DNA sequencing service was provided by first BASE (Singapore).

Plasmid Construction. Oligonucleotides used for plasmid constructions were listed in SI Table S1. To create the platform for multiple-gene integration into yeast chromosomes, the new δ-integration platform was constructed as follows. The δ sequence was amplified from genomic DNA of BY4742, cut with *Hin*dIII/*Eco*RI, and inserted into pUC18, to yield pδ-BLANK. The δ sequence used in the present study was provided in SI Table S5. The plasmid pδ-BLANK was cut with *Xho*I and dephosphorylated by CIP, inserted with the dominant selection marker amplified from pUG66 using primer pair F_BLE_SS and R_BLE_*Sal*I to create pδBLE (Overlapping PCR was performed to remove the internal *Sal*I restriction site of selection marker). Next, the dual promoter pGAL1/10 cassette was amplified from a modified version of pESC-URA (with an additional *Sal*I introduced between the original *Sac*I and *Bgl*II) with primer pair F_tADH1_*Sph*I and R_tCYC1_*Sph*I, digested with *Sph*I and inserted into pδBLE at the same site, to yield pδBLE2.0. Next, mitochondrial targeting sequence from subunit IV of yeast cytochrome c oxidase (encoded by *COX4*)[26] was amplified from BY4742 genomic DNA using primer pair F_NCOX4_*Bam*HI and R_NCOX4_BS, and inserted into pδBLE2.0 cut with *Bgl*II and *Sal*I, to yield pδBLE2.1. For the initial test of genome integration platform, *EGFP* gene was amplified from plasmid pKT127, cut with *Bam*HI/*Xho*I and inserted into pδBLE2.1 cut with the same enzyme pair, to yield pδBLE2.1-EGFP.

For constructing mitochondrion-based expression of isobutanol pathway, intermediate plasmids were constructed as follows. To target KDC and ADH for the expression into mitochondria, *ARO10* and *ADH7* were amplified from BY4742 genomic DNA, and inserted into *Bgl*II/*Sal*I sites of pδBLE2.1. The subsequent constructed plasmids were designated as pδBLE2.1-ARO10 and pδBLE2.1-ADH7. Genes involved in valine biosynthesis pathway, namely, *ILV2*, *ILV5*, and *ILV3*, were also amplified from the genomic DNA of *S. cerevisiae* and inserted into *Bam*HI/*Xho*I site of pδBLE2.1, to yield pδBLE2.1-ILV2, pδBLE2.1-ILV5, and pδBLE2.1-ILV3, respectively. To this end, all isobutanol pathway genes were put under the control of strong galactose inducible promoters (as shown in SI Table S2). These plasmids were next served as templates for the subsequent PCR amplification of genome integration cassettes to achieve combinatorial genome integration of isobutanol pathway genes. Specifically, primer pair F_Delta_Integ/R_pGAL1/10_Integ was used to amplify upstream module and F_pGAL1/10_Integ/R_Delta_Integ was used for the amplification of downstream module, so that the entire isobutanol pathway can be combinatorially integrated into δ-sites of Ty elements.

All eight genes involved in mevalonate pathway were amplified from genomic DNA of *S. cerevisiae* BY4742 using primers listed in SI Table S1. Overlapping PCR was used to remove *Bam*HI site from *ERG12* gene. All PCR products were digested with *Bam*HI/*Xho*I. The digested products of *ERG13*, *ERG12*, *ERG19*, and *IDI1* were inserted into pδBLE2.0 cut with *Bgl*II/*Sal*I, to yield a series of intermediate plasmids. Next, *ERG10*, *tHMG1*, *ERG8*, and *ERG20* were inserted into intermediate plasmids cut with *Bam*HI/XhoI. Subsequently constructed plasmids were named as pδBLE2.0-ERG13/ERG10, pδBLE2.0-ERG12/tHMG1, pδBLE2.0-ERG19/ERG8, and pδBLE2.0-IDI1/ERG20 (as listed in SI Table S2). 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 and primer pair F_Delta_Integ/R_Delta_Integ was used for the subsequent PCR amplification of genome integration cassettes.

For restricting *ERG9* expression in the engineered strain, plasmid pLEU/pCTR3-ERG9 was created as follows. LEU selection marker was amplified from pUG73 with primer pair F_LEU_*Hin*dIII and R_LEU_EB, cut with *Hin*dIII and *Eco*RI, and inserted into pUC18, to generate intermediate plasmid. Next, the promoter region of pCTR3 was amplified from the genomic DNA of *S. cerevisiae* CEN.PK2-1C with primer pair F_pCTR3_*Bam*HI and R_pCTR3_*Eco*RI. The purified PCR product was cut with *Bam*HI and *Eco*RI, and inserted into the intermediate plasmid to yield pLEU/pCTR3-ERG9. This plasmid was then served as template for the PCR amplification of genome integration cassette using primer pair F_ERG9_Integ/R_ERG9_Integ.

Yeast Transformation and Library Construction. For library construction, electroporation was performed as follows. YPD medium (10 mL) was inoculated with overnight *S. cerevisiae* BY4742 culture to an initial OD_{600} of 0.3, Yeast cells were harvested by centrifugation at 4 °C, 3000 rpm for 5 min after 4−5 h when OD_{600} reached 1.2. The cell pellet was washed twice with 10 mL ice-cold Milli-Q water, followed by centrifugation to collect cells. Next, cells were washed with 1 mL ice-cold 1 M sorbitol, pelleted by centrifuge and finally resuspended in ice-cold sorbitol to a final volume of 50 μL. Subsequently, 50 μL of yeast cells together with approximately 10 μg mixture of equimolar individual integration cassette was electroporated in a 0.2 cm cuvette at 1.6 kV. After electroporation, cells were immediately mixed with 3 mL prewarmed YPD medium and shaken 2−3 h on a rotary shaker to recover cells. Following that, cells were collected by centrifugation at 3000 rpm for 5 min on a centrifuge, washed and resuspended in ddH_2O. For the construction of isobutanol pathway library, appropriate amount of cells was plated on YPD plate supplemented with 40 μg/mL, 80 μg/mL, and 160 μg/mL of phleomycin (InvivoGen, San Diego, U.S.A.). Overall, three libraries for isobutanol biosynthesis were constructed with cells adapted to different concentrations of phleomycin. For the

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construction of mevalonate pathway library, cells were directly spotted on YPD agar plate supplemented with even higher concentration of phleomycin to a final concentration of 240 μg/mL, to achieve integration of the entire mevalonate pathway into yeast chromosomes.

To generate strains with *ERG9* under control of copper repressible promoter pCTR3, the preparation of competent cells and electroporation was carried out in a similar way as mentioned above except that cells were mixed together the genome integration cassette amplified from pLEU/pCTR3-ERG9 with primer pair F_ERG9_Integ and R_ERG9_Integ.

PCR Verification of Genome Integration Events. For the subsequent verification of genome integration events, colonies were first picked up from the library and streaked on phleomycin containing plates to eliminate the false positive strains. Next, universal primer F_pGAL1Scr/R_tCYC1Scr and F_pGAL10Scr/R_tADH1Scr were used for the PCR verification of genome integration of pathway genes. Cells were lysed by 20 mM NaOH for 15 min in 100 °C water bath. PCR program was set as follows: 1 cycle of 95 °C for 5 min; amplification, 30 cycles of 95 °C for 15 s, 50 °C for 30 s, and 68 °C for 90 s; 1 cycle of 68 °C for 3 min.

For isobutanol-producing strains, the band size of 2.0 kb and 1.2 kb for the first PCR reaction with primer pair F_pGAL10Scr/R_tADH1Scr corresponded to *ARO10* and *ADH7*, respectively. The band size of 2.1 kb, 1.8 kb, and 1.2 kb for the second PCR reaction with primer pair F_pGAL1Scr/R_tCYC1Scr corresponded to *ILV2*, *ILV3*, and *ILV5*, respectively. For mevalonate pathway library, bands with size of 1.5 kb, 1.3 kb, 1.2 kb, and 900 bp for the PCR reaction with primer pair F_pGAL10Scr/R_tADH1Scr corresponded to *ERG13*, *ERG12*, *ERG19*, and *IDI1*. Band with size of 1.6 kb, 1.35 kb, 1.2 kb, and 1.0 kb for the PCR reaction with F_pGAL1Scr/R_tCYC1Scr corresponded to *tHMG1*, *ERG8*, *ERG10*, and *ERG20*, respectively.

Isobutanol Production in Engineered Yeast Cells. For the characterization of isobutanol production in the engineered yeast strains, small-scale study was carried out in 14 mL conical tubes. Specifically, conical tubes containing 2 mL SC medium (3.8% galactose and 0.2% glucose) with valine dropped out were inoculated to an initial OD_{600} of 0.05 with overnight cultures. Alcohol levels were measured after 4 days of cultivation. Cell culture (100 μL) was aliquoted for measuring OD_{600} by microplate reader (Synergy H1, BioTek, U.S.A.). Alcohol compounds were isolated by solvent extraction. Supernatant of culture broth after centrifugation (500 μL) was extracted with 1 mL GC standard grade toluene spiked with *n*-butanol. Organic phase (900 μL) was next diluted with 900 μL of toluene and subjected to GC-MS analysis. For GC-MS analysis, 1 μL of diluted sample was injected into Shimadzu QP2010Ultra system equipped with a DB5 ms column (30 m × 250 μm × 0.25 μm thickness) (Agilent Technologies, U.S.A.). Split ratio was set with 15:1. Helium (ultrapure) was used as carrier gas at a flow rate 1.0 mL/min. GC oven temperature was initially held at 40 °C for 2 min, ramped with a gradient of 5 °C/min until 45 °C and held for 4 min. Then, it was raised with a gradient 15 °C/min until 230 °C and held for 4 min. The injector and detector were maintained at 225 °C. Scan mode was used to detect mass range 40−120 m/z. For the quantitation of isobutanol produced by engineered strains, authentic alcohol compound from Sigma-Aldrich was used for plotting standard curve. To correct for differences during sample preparation, the internal spiked *n*-butanol was used for normalizing the values.

Amorpha-4,11-diene Production in Engineered Yeast Cells. Engineered strains were transformed with pYES2ADS or pRS416ADS through conventional lithium acetate approach. Small-scale studies were carried out for amorpha-4,11-diene production. Specifically, 14 mL sterile round-bottom tubes containing 2 mL SC medium (1.8% galactose and 0.2% glucose) with uracil dropped out were inoculated to an initial OD_{600} of 0.05 with overnight cultures. For studying copper-mediated repression of *ERG9* to minimize ergosterol biosynthesis, 150 μM of $CuSO_4$ were added into culture media. All tubes were immediately added with 200 μL dodecane after seeding, to perform two phase fermentation and harvest amorpha-4,11-diene. Both amorpha-4,11-diene production and cell density were monitored after 5 days. 100 μL of cell culture was taken for measuring OD_{600} by microplate reader, and 10 μL dodecane layer was sampled and diluted in 990 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 DB5 ms column (Agilent Technologies, U.S.A.). Helium 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, 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 40−240 m/z. For the quantitation of amorpha-4,11-diene level, amorpha-4,11-diene was quantitated by GC-MS and FID. Caryophyllene was used for plotting the standard curve, and amorpha-4,11-diene levels in the present study represented as caryophyllene equivalents.

RNA Extraction and qRT-PCR. Fresh overnight cell culture was inoculated into 2 mL SC media to an initial OD_{600} of 0.05. Cells were cultured on a rotary shaker for 12 h and harvested during the exponential growth phase. Approximately 1×10^7 cells were collected by centrifugation and washed twice with phosphate saline buffer. Next, the total RNA was extracted using RNeasy Mini Kit (QIAGEN, Germany). During RNA extraction, genomic DNA contamination was eliminated by in-column digestion with DNase I (QIAGEN, Germany). The concentration of eluted RNA solution was quantified by measuring the absorbance at 260 nm. Approximately 500 ng of RNA was converted to cDNA using iScript Reverse Transcription Supermix from Biorad.

Oligonucleotides for qRT-PCR studies of pathway genes and actin (*ACT1*, internal reference gene) were designed using the ProbeFinder (https://www.roche-applied-science.com), and oligonucleotides used for qRT-PCR experiments were listed in SI Table S3. qRT-PCR analysis was performed using LightCycler 96 real-time machine with FastStart Essential DNA Green Master 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: preincubation, 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*.[30] All assays were performed in triplicate, and the reaction without reverse transcriptase was used as a negative control.

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■ ASSOCIATED CONTENT

Supporting Information

This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Tel: +65 66013179. Fax: +65 67764382. Email: cheyuan@nus.edu.sg.

Author Contributions

J.Y. designed and performed experiments. J.Y. and C.B.C. wrote the manuscript.

Notes

The authors declare no competing financial interest.

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