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Building Terpene Production Platforms in Yeast

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ABSTRACT: Plants and microbes commonly make terpenes and terpenoids in small amounts and as complex mixtures, and their chemical synthesis is often costly and inefficient. Hence, there are many efforts to create robust and efficient biological production platforms for this interesting class of molecules. In this study, our effort was directed towards building a yeast production platform using an unbiased genetic selection approach. Yeast strain BY4741 was subjected to EMS mutagenesis, followed by selection for growth in the presence of nystatin, squalestatin, and exogenous cholesterol. This unbiased screen selected for mutant yeast lines having a dispensable mevalonate pathway and containing uncharacterized *SUE* (sterol uptake enhancement) mutations supporting aerobic uptake of exogenous sterol. These mutants were next screened for high level accumulation of farnesol (FOH), an indicator for high level accumulation of the key intermediate FPP, farnesyl diphosphate. To further improve the FPP pool in these mutants, insertional mutations into the *ERG9* gene (coding for squalene synthase) were introduced into those lines capable of accumulating $\geq$50 mg farnesol/L. This generated another series of lines that accumulated farnesol levels over 70 mg/L in small-scale shake cultures. To evaluate the utility of these lines as a general production platform for specific terpenes, select *SUE*/*erg9* lines were transformed with a vector harboring the *Hyoscyamus muticus* premnaspirodiene synthase (*HPS*) gene encoding for a sesquiterpene synthase. The new yeast line ZX178-08 accumulated the highest level of premnaspirodiene, up to 116 mg/L, with FOH levels of 23.6 mg/L. In comparison, the parental line BY4741 accumulated 10 times less premnaspirodiene, 10.94 mg/L, with no farnesol detectable. Co-expression of the *HPS* gene with an amino-terminal truncated, catalytic form of the hamster *HMGR* gene, *tHMGR*, increased premnaspirodiene accumulation to 170.23 $\pm$ 30.44 mg/L, almost a 50% increase. Further utility of this yeast line was demonstrated for triterpene production. When engineered for the production of a non-native triterpene, Zx178-08 accumulated upwards of 60 mg/L of botryococcene. To engineer more native triterpene accumulation, additional insertion mutants into the *ERG1* gene (coding for squalene epoxidase) were evaluated. Insertion of a simple selection marker followed by over-expression of a heterologous squalene synthase gene resulted in greater than 85 mg/L of squalene. However, when the *ERG1* insertional mutant included chromosomal insertion of a truncated, heterologous *HMGR* gene, squalene production was more than tripled to 270 mg/L. These results are discussed in comparison to other recently developed terpene production platforms.

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Terpenes are a very large class of structurally diverse compounds made by organisms in all kingdoms of life. The terpenes from plants are perhaps the most extensively described as evident by well over 100,000 different terpenes reported in the literature (Buckingham, 2003). Terpenes are also widely recognized for their diverse utility and applications with significant market values in medicines, flavors and fragrances, agricultural chemicals, and industrial chemicals. For example, taxol, a diterpene widely recognized for its application as a chemotherapeutic agent, was first isolated from the bark and needles of several Taxus plant species (Wall and Wani, 1995), now widely used to treat patients with lung, ovarian, breast, and brain cancers, and reached peak annual sales of US$ 1.6 billion in 2000 (Malik et al., 2011; Yared and Tkaczuk, 2012). Likewise, Artemisinin, a sesquiterpene isolated from the plant *Artemisia annua*, has been developed as a key pharmacological agent for the control of malaria (Tu, 2011), with annual global demand exceeding 100 metric tons in 2006 (Kindermans et al., 2007). Patchouli, another sesquiterpene, is a popular aromatic found in colognes, perfumes and many other household cleaning products (Wu et al., 2006). The current world demand for patchouli is 1,200–1,500 tons/year with a 10–15% increase per year predicted (Ramya et al., 2013). Menthol is a monoterpene obtained from mint family plants and is a popular ingredient in many foods and consumer products (Bedoukian, 1995). Triterpenes such as squalene, obtained from various plant sources and the livers of deep sea sharks, have utility as a nutraceutical product, are used extensively in many types of cosmetics, and have special application as a lubricant for high performance machinery (Bhilwade et al., 2010; Huang et al., 2009; Reddy and Couvreur, 2009).

Although there is a large demand for specific terpenes, the availability of single terpene entities is limited in nature. Terpenes are generally made natively by plants and microbes in small amounts and components of complex mixtures that vary with

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growth and environmental conditions, making it difficult to reproducibly obtain large amounts of any one terpene constituent (Wu et al., 2006). For instance, taxol was initially produced directly from the bark of Pacific yew in a process that killed the producing trees. Now most taxol is derived from a semi-synthetic process wherein the basic backbone structure is obtained from plant cell fermentation, followed by chemical conversion to the final product (Jennewein and Croteau, 2001). Even though there is still ambiguity about the biosynthetic pathway for taxol, many efforts have been devoted to improving the production of intermediates, precursors, and derivatives via metabolic engineering techniques to provide alternative sources to meet the worldwide market demand for this and other therapeutically classes of compounds (Ajikumar et al., 2010; Besumbes et al., 2004; Boghigian et al., 2012; Engels et al., 2008).

Organic chemical synthesis does not suffice for the production of single entity terpene compounds to satisfy the market requirement. Chemical synthesis of terpenes is often costly and inefficient (Nicolaou et al., 1994). Furthermore, chemical synthesis also suffers from generating enantiomeric mixtures, which adds other complications and difficulties for downstream application to separate and purify, if one particular stereochemical form of a terpene is desired. Given such difficulties, there are many on-going efforts to create robust, reliable and efficient biological systems for the production of distinct classes of terpenes, and more so for the generation of stereochemically pure forms of terpenes (Asadollahi et al., 2008,2010; Fischer et al., 2011; Keasling, 2009; Kirby et al., 2008; Martin et al., 2003; Paddon et al., 2013; Seki et al., 2008; Takahashi et al., 2007; Wu et al., 2006).

In the current effort, we have taken a combination of genetic and molecular genetic approaches to engineering a dispensable mevalonate biosynthetic pathway in yeast. Our approach has been to recognize that this pathway provides allylic diphosphate precursors, DMAPP, IPP, FPP, and GGPP, for a number of necessary and essential biosynthetic needs. For instance, DMAPP and IPP for prenylation of macromolecules like tRNAs, FPP for ergosterol biosynthesis and protein prenylation, and GGPP for dolichol biosynthesis and other protein prenylation reactions. Our approach has been to generate a sterol auxotrophic line with unregulated flux of carbon through the mevalonate pathway sufficient to provide DMAPP, IPP, FPP, and GGPP for essential biosynthetic needs, yet robust enough such that carbon flux can be diverted at any of these intermediates to build additional novel terpene molecules. The aim of the current study was to engineer yeast for high carbon flux down the mevalonate pathway sufficient to enhance the accumulation of FPP for the efficient and economic production of sesquiterpene and triterpene products sufficient to meet the requirements of research, industrial production, or therapeutic applications.

Materials and Methods

Chemical and Media Preparations

All chemical reagents were obtained from Sigma–Aldrich (St. Louis, MO), BD Bioscience (Franklin Lakes, NJ), or Fisher Scientific (Chicago, IL), while reagents for molecular manipulations were from Stratagene (San Diego, CA), Takara (Shiga, Japan), Invitrogen (San Diego, CA), and New England Biolab (Ipswich, MA).

Bacteria and yeast were grown using standard culture practices as described in the supplementary information.

Ethyl Methane-Sulfonate (EMS) Mutagenesis

Strain BY4741 (*MAT**a** his3*Δ0, *leu2*Δ0, *met15*Δ0, *ura3*Δ0) (Janke et al., 2004) was used as the parental yeast line. BY4741 cells were incubated overnight at 30 °C in 5 mL YPD medium with shaking at 200 rpm, and used to establish a 200 mL YPD shake flask culture. When the yeast culture OD_{600} reached approximately 1.0, the cells were spun down by centrifugation (10 min at 4,000 × *g*), and washed twice with 20 mL 0.1 M sodium phosphate buffer, pH7.0. Cells were concentrated by centrifugation again, re-suspended in 1 mL 0.1 M sodium phosphate buffer, transferred to a 14 mL FALCON culture tubes, treated with 300 μl EMS (1.2 g/mL), followed by incubation at 30 °C for 1 h with shaking. To stop the mutagenesis, 8 mL of sterile 5% sodium thiosulfate was added to yeast cells to inactivate the EMS. Cells were pelleted, washed with 8 mL sterile water, concentrated by centrifugation, re-suspended in 1 mL sterile water and 100 μl aliquots plated onto YPD-NCS agar plate (YPD plus 50 mg/L cholesterol, 50 mg/L nystatin, 50 mg/L squalestatin, 2% Bacto-agar). In some experiments, the washed cells were resuspended in 1 mL YPDE liquid media for recovery overnight before plating on YPD-NCS agar medium. The cultures were incubated for up to 2 weeks at 30 °C until distinct colonies became visible.

Yeast Transformation and Colony Screens

Yeast strains were transformed with the respective vector constructs using the FROZEN-EZ Yeast Transformation II Kit (Zymo Research, Orange, CA) according to the manufacturer's recommendations. Resulting colonies were cultured and chemically profiled by GC-MS using standard protocols as described in the supplementary information.

GC-MS Detection and Quantification of Terpenes

Extraction and chemical profiling by GC-MS of the various yeast cultures were preformed as described previously (Takahashi et al., 2007) and detailed in the supplementary information.

Construction of the Squalene Synthase *(ERG9)* and Squalene Epoxidase *(ERG1)* Knockout Mutations

Insertional mutations into the *ERG9* and the *ERG1* genes relied on standard procedures as described more fully in the Supplementary Information. Essentially, DNA sequences at either ends of the target loci were appended to either a hygromycin selection marker (for *ERG*9 KO), or the *KanMX4* marker or the *KanMX4* gene plus truncated *HMGR* gene (for *ERG1* KO), and these combined DNA fragments amplified by PCR were transformed into our target yeast lines followed by the selection for the indicated antibiotic markers. The insertional mutants were confirmed by PCR and then chemically profiled by GC-MS.

Expression of Terpene Synthases and HMGR Genes in Yeast

The various expression vectors used in these studies are illustrated in Figures S2 and S3 in the supplementary information. The yeast *GPD* promoter (P-*GPD*), also referred to as *TDH3* promoter, was amplified from the pYM-N14 plasmid described by (Janke et al., 2004) using the primers *GPD-Bam*HIF and *GPD-Not*IR primers and inserted into the pESC-*HIS3* vector (Agilent Technologies, Santa Clara, CA) digested with *Bam*HI and *Not*I to replace the original *GAL1/10* divergent promoters. The resulting plasmid was named pESC-*HIS3-GPD*. The *HPS* gene was cloned into *Not*I and *Spe*I sites of pESC-*HIS3-GPD* to obtain the yeast expression vector pESC-*HIS3-GPD-HPS* similar to that described by Takahashi et al. (2007) (Fig. S2). The pESC-*LEU2-GPD* vector was built in the same way as pESC-*HIS3-GPD* vector. The hamster *HMGR* full-length and truncated *HMGR* genes (Chappell et al., 1995) were cloned into *Not*I and *Spe*I sites of pESC-*LEU2-GPD* to obtain the yeast expression vector pESC-*LEU2-GPD-tHMGR* and pESC-*LEU2-GPD-HMGR* full constructs. *The Botryococcus braunii* squalene synthase and botryococcene synthase (*SSL1-3*) expression constructs were built similarly using the corresponding genes described in (Niehaus et al., 2011) and illustrated in Figure S3. Yeast lines transformed with these constructs were then evaluated for their production of the sesquiterpene premnaspirodiene and the triterpenes squalene and botryococcene as a measure of the availability of intermediates of the mevalonate biosynthetic pathway for the biosynthesis of new terpenes.

Results

Overall Strategy to Generate New Yeast Strains for Terpene Production

Figure 1 outlines the approach used to generate yeast lines providing the robust terpenoid biosynthesis of precursors that can be utilized for the production of many different classes of terpenes. The strategy takes advantage of the native mevalonate (MVA) pathway that operates normally in yeast for the biosynthesis of ergosterol, the dominant sterol found in yeast. Ergosterol is the main product of the yeast mevalonate pathway, is an important membrane component, and is essential for yeast growth. If the ergosterol biosynthetic pathway is blocked or inhibited, yeast die. In fact, this is the basis for many pharmacological drugs to control fungal infections in man (Maertens, 2004) and agricultural chemicals to control fungal infection in plants (Casida, 2009). To further complicate matters, wild type yeast can take up exogenously supplied sterol from their environment only under anaerobic conditions (Zavrel et al., 2013).

In order to efficiently channel terpene biosynthetic intermediates from the ergosterol biosynthetic pathway, a *SUE* (sterol uptake enhancement) mutation supporting the aerobic uptake and utilization of exogenous sterol was first created (Bourot and Karst, 1995; Shianna et al., 2001). A *SUE* mutation is thus a yeast line that can meet all its sterol needs by an exogenous source of sterol, thereby making the endogenous ergosterol biosynthetic pathway dispensable. The *SUE* mutation was then complemented by the introduction of a knockout mutation in the *ERG9* gene (squalene synthase) (Zhang et al., 1993), resulting in a yeast line where the MVA pathway was still operational for the biosynthesis of FPP and downstream allylic intermediates like geranylgeranyl diphosphate (GGPP), yet intermediates in the pathway (DMAPP, IPP, and FPP) could be diverted to the biosynthesis of other non-essential terpene components. In order to follow and select for the desired mutant lines, the yeast lines could be monitored for farnesol (FOH) accumulation, the dephosphorylated form of farnesyl diphosphate as noted earlier by Song et al. (2003). If the MVA pathway in the yeast line continued to operate as proposed, then one would expect carbon flux to FPP to continue. But, because the downstream utilization of FPP by squalene synthase was abolished, then the accumulating FPP would be subject to the endogenous phosphatase activity (Faulkner et al., 1999) for its conversion to FOH, which could be used as an initial screen for monitoring development of the mutant yeast line. Further engineering of such a yeast line could then take advantage of the FPP, DMAPP, and IPP pools for their diversion to the biosynthesis of monoterpenes (10 carbon compounds), sesquiterpenes (15 carbon compounds), diterpenes (20 carbon compounds), and triterpenes (30 carbon compounds).

Steps in the Development of Yeast With a Dispensable Mevalonate Pathway

To initiate the new yeast line development, we subjected parental yeast line BY4741 through three phases of selection. Figure 2 illustrates these three phases. In phase I, chemical mutagenesis was used to introduce *SUE* mutations, which were identified by selecting for yeast cells that did not have a functioning ergosterol biosynthetic pathway and could only grow in the presence of exogenous cholesterol. The *SUE* mutation was created by subjecting wild type yeast strain BY4741 to EMS mutagenesis to introduce random mutations in the whole genome, followed by selection on plates containing three selection agents: nystatin; cholesterol; and squalestatin. Nystatin binds to ergosterol in the cell membrane causing non-selective membrane permeability and leads to cell death (Silva et al., 2006). Nystatin thus selects against cells that have ergosterol in their membranes. However, yeast has an absolute requirement for sterols in order for their membranes to function properly. Hence, by having the mutagenized yeast plated in the presence of cholesterol, which nystatin cannot bind to, only yeast that can take up the exogenous cholesterol under aerobic conditions and properly incorporate the cholesterol into their membranes survive. Squalestatin is a potent inhibitor of squalene synthase and eliminates the yeast's ability to synthesize ergosterol (Bergstrom et al., 1995), thus assuring that the surviving yeast have a dispensable mevalonate pathway.

Two hundred of the initial yeast colonies growing aerobically after mutagenesis on medium containing nystatin, cholesterol and squalestatin were replicate plated onto medium containing squalestatin or squalestatin and ergosterol. One hundred and thirty-four of the colonies grew on both plates, and only 66 of the colonies re-grew on the squalestatin plates supplemented with ergosterol but did not grow on the squalestatin only plates. This indicated that only 33% of the initial 200 colonies picked exhibited the desired phenotype.

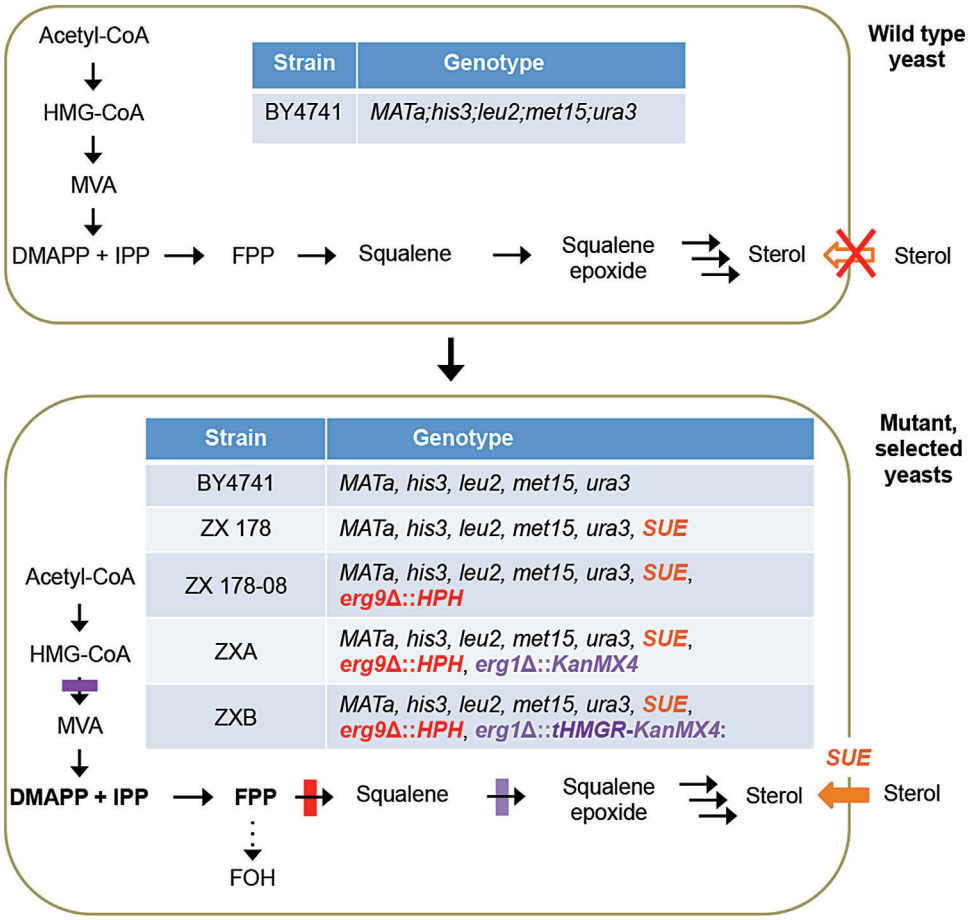

Figure 1. Overall approach for generating yeast lines that would have a dispensable sterol biosynthetic pathway, and would provide opportunities for diverting intermediates (DMAPP, IPP, and FPP) from the mevalonate (MVA) pathway for the biosynthesis of other terpene compounds.

In phase II, the 66 yeast lines demonstrating an absolute requirement for exogenous sterols for growth were grown in liquid medium containing squalestatin plus ergosterol, and chemically profiled by GC-MS after 6 days (Fig. 3). Like the parental line BY4741, 20 of the culture lines did not accumulate detectable amounts of farnesol. Forty-six of the cultures accumulated 1–60 mg of FOH/L equivalent of culture, with nine of these accumulating more than 50 mg FOH/L. Eight of mutant lines accumulating 50 or more mg/mL of FOH were carried forward for phase III knockout mutagenesis of the squalene synthase gene, *ERG9*.

The objective in phase III was to obtain a knockout mutation of the *ERG9* (squalene synthase) gene, thus assuring the dispensable nature of the endogenous mevalonate pathway for ergosterol biosynthesis. Site specific recombination was afforded by appending 5' and 3' regions surrounding the native *ERG9* gene onto a hygromycin selection marker gene, then introducing this linear gene construct into each of the selected yeast lines from the phase II screening under conditions to promote site-specific, double recombination with the native *ERG9* gene. The knockout mutants were then selected by plating the cells in the presence of ergosterol and hygromycin. Recombination as depicted in Figure 2 should result in the coding region of the *ERG9* gene being substituted with the hygromycin resistance marker gene. Replica plating of the resulting hygromycin resistant colonies onto plates plus and minus for ergosterol confirmed that only *erg9* knockout colonies grew with sterol supplement, and did not grow on plates without sterol supplement. Confirmation of the *ERG9* substitution was obtained by screening the genomic DNA of the selected sterol dependent yeast colonies for the hygromycin marker gene in proximity to genomic DNA sequences normally found 3' to the *ERG9* coding region. Using genomic DNA isolated from hygromycin resistant colonies as the PCR templates with a hygromycin specific primer (*hph*F) and a primer specific to a genomic DNA sequence found 3' to the *ERG9* gene (*ERG9* 450DwR), a PCR amplification product of approximately 1,500 bp would be expected and was readily evident in the colonies tested (Fig. 4A). A third level of *ERG9* knockout was provided by chemically profiling the selected lines for their accumulation of farnesol when grown in small-scale liquid cultures. As noted previously, the parental line BY4741 does not accumulate farnesol (the dephosphorylated product of FPP), but the selected lines, like ZX178-08 typically accumulated greater than 100 mg of FOH/L equivalent as determined by GC-MS (Fig. 4B).

Eight independent colonies from each of the *erg9* knockout lines were then re-evaluated for their growth in liquid media and the

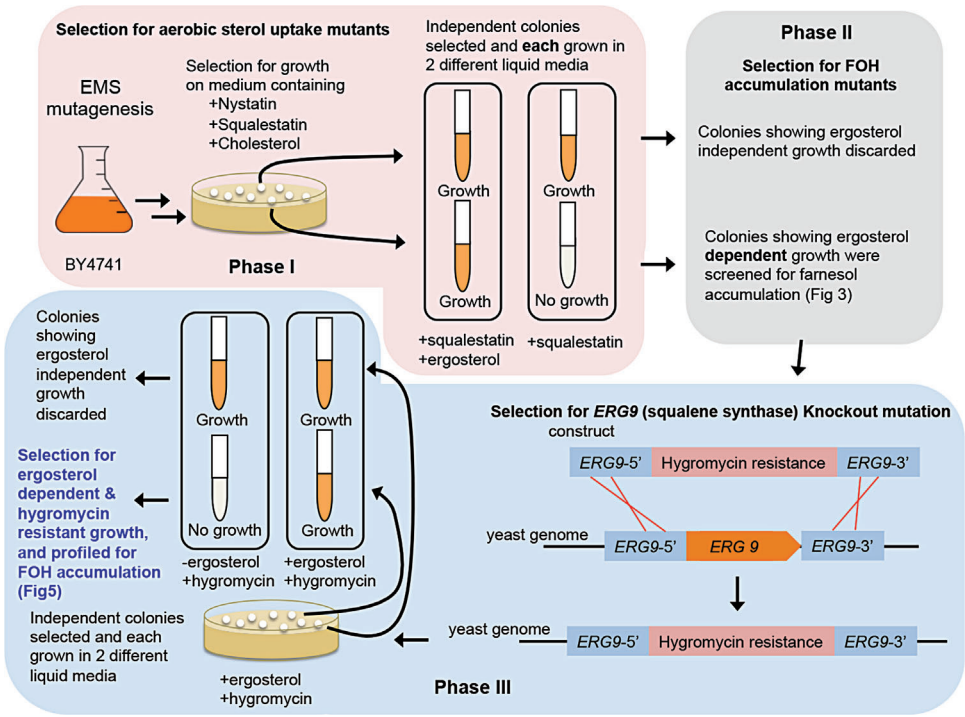

Figure 2. Three phases in selection of yeast lines dependent upon exogenous sterols for aerobic growth and having a dispensable mevalonate biosynthetic pathway.

dispensable nature of their mevalonate pathway by checking for their accumulation of farnesol (Fig. 5). Surprisingly, upwards of twofold variation in the levels of farnesol accumulation were observed in some of the eight independent cultures from a single *erg*9 knockout line. For example, line ZX178-05 accumulated 32 mg FOH/L while line ZX178-03 accumulated ~79 mg FOH/L. Twenty percent variation in terpene production is not unusual in experimental replicates of cultures arising from the same initial yeast colony, but greater than 20% suggested additional genetic and epigenetic alterations that could be segregating, separating and manifesting in these populations. Nonetheless, 11 of the resulting yeast lines accumulated more than 70 mg/L farnesol in test tube scale cultures and were targeted for qualification of novel terpene production ability.

Qualification of the New Mutant Yeast Lines for Their Utility to Produce Terpene Compounds

Sesquiterpene Production

Eight of the yeast lines harboring a *SUE* mutation and having the native *ERG9* gene deleted in one selection series (Fig. 5, the ZX series) and another line derived from an identical, but independent selection series (line 2–2) were evaluated indirectly for the available of terpene biosynthetic intermediates, and specifically FPP, to support sesquiterpene biosynthesis in comparison to the parental strain BY4741 (Fig. 6A). *Hyoscyamus* premnaspirodiene synthase (*HPS*), a catalytically active sesquiterpene synthase first isolated from *Hyoscyamus muticus*, was chosen for this evaluation because *HPS* has been characterized for its expression in bacteria (Mathis et al., 1997) and in yeast (Takahashi et al., 2007). An appropriate *HPS* gene expression vector was engineered into the indicated yeast lines and the subsequent transformants screened for premnaspirodiene accumulation when the yeast were grown as 30 mL shake flask cultures with SCE media containing leucine, tryptophan, uracil, and methionine for 12 days at 23 °C. Yeast line ZX178-08 accumulated the highest level of premnaspirodiene, up to 116 ± 23.64 mg/L, with FOH levels of 23.6 ± 14.5 mg/L. In comparison, the parental line BY4741 accumulated 10 times less premnaspirodiene, 10.94 ± 3.12 mg/L, with no farnesol accumulation detected.

HMGR (3-hydroxy-3-methylglutaryl coenzyme A reductase) catalyzes the irreversible conversion of 3-hydroxy-3-methylglutaryl coenzyme A to mevalonate and is considered a key regulatory step controlling sterol biosynthesis metabolism in all eukaryotic organisms (Siperstein and Guest, 1960). The 5' end of HMG CoA Reductase gene encodes for sequences responsible for tethering the HMGR enzyme to the endoplasmic reticulum and mediating a regulatory network controlling sterol-mediated inhibition of *HMGR* gene transcription (Gil et al., 1985). To engineer elevated HMGR enzyme activity into yeast and further advance terpene product engineering, a truncated and full-length version of the hamster HMG-CoA reductase gene were co-expressed with *HPS* gene in the newly developed yeast line ZX178-08. The truncated hamster *HMGR* was produced by removing the DNA sequences encoding for the regulatory and N-terminal,

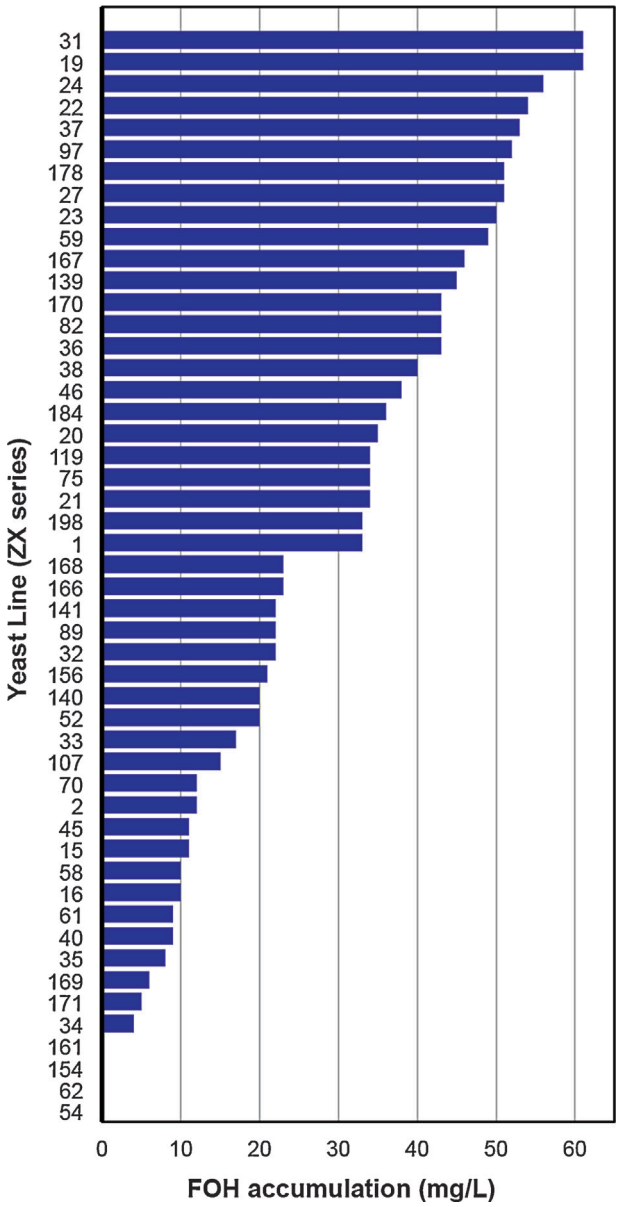

Figure 3. Quantitation of farnesol (FOH) levels in yeast lines demonstrated to have an exogenous sterol requirement for growth. Selected yeast lines were grown as test tube shake cultures with 40 μg/mL of ergosterol and 40 μg/mL of squalestatin for 6 days prior to chemically profiling culture aliquots by GC-MS.

membrane-spanning domains (Chin et al., 1985). Constitutive expression of this truncated form of the hamster *HMGR* gene in transgenic tobacco was previously shown to lead to a three- to sixfold increase in the total *HMGR* enzyme activity and a three- to tenfold increase in total sterol accumulation (Chappell et al., 1995). Co-expression the *HPS* gene plus the full length *HMGR* gene in yeast line ZX178-08 improved premnaspirodiene production to 132 ± 11.5 mg/L with 31.2 ± 7.2 mg/L FOH (Fig. 6C). In contrast, co-expression of *HPS* gene along with truncated *HMGR* gene resulted in premnaspirodiene production of 170.2 ± 30.4 mg/L with 21.88 ± 3.82 mg/L farnesol, almost an increase of 50 mg of premnaspirodiene per liter relative to line ZX178-08 with the *HMGR* gene (Fig. 6B). Also shown in Figure 6B and C are the culture growth curves, which are approximately half the growth rate of that for the parental line BY4741, which reached the same culture saturation level within 5–6 days rather than 10–12 days. About 50–80% of the total premnaspirodiene and farnesol produced was detected in the culture medium, suggesting that the newly development yeast strain might have secreted these terpenes, or that these particular terpenes possibly diffused down a concentration gradient, or were released upon cell lysis.

Triterpene Production

A further demonstration of the dispensable nature of the mevalonate (MVA) pathway in strain ZX178-08 was sought. The potential to divert accumulating FPP as a consequence of cell culture growth towards the biosynthesis of other potential high-valued triterpenes was thus examined. Botryococcene is a squalene-like triterpene found only in the green algae *Botryococcus braunii* race B (Okada et al., 1995), whose production in yeast would not likely be subject to catabolism. Botryococcene is a direct precursor to all classes of combustible fuels, gasoline, jet fuel and diesel (Hillen et al., 1982). We hence introduced a chimeric construct consisting of fusing squalene synthase-like genes *SSL-1* and *3* into ZX178-08. *SSL1-3M* consists of the *SSL1* and *SSL3* genes fused with a triplet repeat linker of GGSG in-between the two genes, plus a 71 amino acid membrane-spanning domain of the carboxyl terminus of the *Botryococcus braunii* squalene synthase according to Niehaus et al. (2011). The *SSL-1* gene encodes for a pre-squalene diphosphate synthase, which catalyzes the conversion of FPP to the cyclopropyl intermediate pre-squalene, a relatively unusual and unstable intermediate. When coupled with the expression of *SSL-3*, pre-squalene is converted into botryococcene in a NADPH dependent manner. Previous work demonstrated that the fusion of these two genes into *SSL1-3* resulted in more efficient biosynthesis of botryococcene (Niehaus et al., 2011). This gene fusion under the control of a moderately active *ADH*1 promoter was thus transformed into yeast ZX178-08 line to evaluate triterpene production capacity for botryococcene (Fig. 7). Similar to premnaspirodiene production, botryococcene accumulation paralleled cell culture growth for the first 4 days, after which the accumulation rate decreased by half. Interestingly, this occurred as the cell cultures were reaching their maximum density, followed by a rather unusual decline, perhaps indicative of a toxicity of the accumulating botryococcene. Like sesquiterpene accumulation, the botryococcene accumulated predominately (>70%) in the culture medium.

In contrast to botryococcene, production of squalene, another linear, branched chain triterpene, could be subject to metabolism by the innate sterol biochemical machinery. Hence, we first sought to inactive squalene epoxidase, the key enzymatic step committing squalene to sterol biosynthesis. A second reason for wanting to inactive the squalene epoxidase enzyme relates to another interesting observation. That is, while our yeast squalene synthase knock out line (*erg9*) can be transformed with the homologous yeast gene and thus complement the *erg9* mutation, we have not been able to obtain yeast transformants harboring other plant or animal

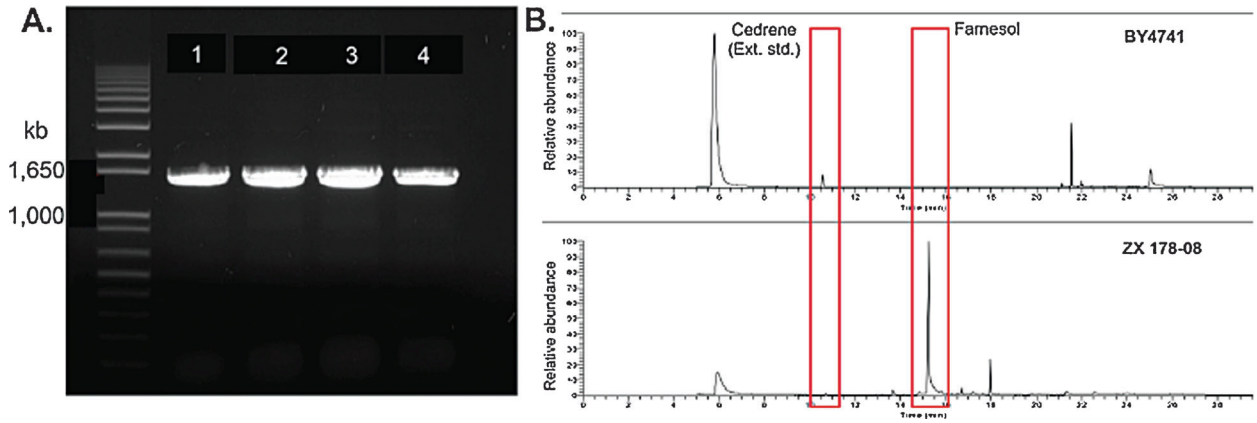

Figure 4. Molecular and chemical confirmation of the *erg9* knockout mutation. DNA isolated from four independent colonies selected for substitution of the hygromycin resistance (*HPH*) gene for the *ERG*9 gene were used as PCR templates with an *HPH* specific primer and a primer specific for the genomic DNA surrounding the *ERG*9 locus (A). If the *HPH* gene did insert and replace the *ERG9* gene, the expected amplification product would be 1,538 bp. Farnesol accumulation in a yeast line (ZX178-08) selected for a dispensable mevalonate biosynthetic pathway plus *erg*9 knock out in comparison to that accumulating in the parental line (BY4741) used to generate the new mutant yeast lines (B). GC chromatographs of hexane extracts prepared from BY4741 and ZX178-08 are compared with peak identities confirmed by MS (data not shown).

squalene synthase genes under transcriptional control of a constitutive promoter. However, when those plant or animal squalene synthase genes are under the control of inducible promoters, like the *Gal*10 promoter, then transformants of ZX178-08 could be obtained and squalene accumulation could be induced upon addition of galactose. We have simply assumed that when squalene is constitutively produced by the foreign plant and animal squalene synthases, the resulting squalene can be convert to 2–3' epoxidosqualene or other downstream derivatives in a manner that would be toxic to the cells. Based on these two reasons, we sought to delete the squalene epoxidase gene (*ERG1*) to enhance the prospects of triterpene engineering.

Insertional inactivation of the *ERG1* gene was created by introducing a fragment containing the G418 resistance gene (*KanMX4)* flanked by 34 bp fragments of the LoxP recombinase system sandwiched (to allow for future recovery of the selection marker) between 400 bp fragments of the 5' and 3' *ERG1* gene (Fig. S4). Introduction of this sequence into ZX178-08 and subsequent selection yielded many G418 resistant colonies. Yeast having correct homologous recombination between the transformed fragment and desired regions of the *ERG1* coding sequence were further confirmed by PCR amplification with primers outside the recombination region (Fig. S4) and this series of yeast were named ZXA. The gene encoding for truncated, catalytic active hamster *HMGR* (HMG-CoA reductase) (Chin et al., 1985), a rate limited enzyme of MVA pathway, was also integrated into *ERG1* gene region along with *KanMX4* selection cassette, and the G418 selected lines referred to as ZXB. The ZXB lines were confirmed by PCR similar to that for the ZXA lines (Fig. S4).

Yeast lines ZXA and ZXB where then transformed with an expression cassette harboring the *B. braunii* squalene synthase gene (Okada et al., 2000) driven by the strong constitutive *GPD* promoter (Fig. S3). While other plant, animal and fungal squalene synthase genes have been evaluated, the algal squalene synthase appears to be catalytically superior in terms of kinetic efficiency (*Kcat/Km*), and hence greater squalene accumulation (Niehaus et al., 2011; Okada et al., 2000). For comparison, FOH accumulation by untransformed ZXA (Fig. 8A) and ZXB (Fig. 8B) was determined over a 12-day cultivation cycle. FOH accumulated to over 120 mg/L in ZXA, and upwards of 300 mg/L in ZXB. When transformed with the algal squalene synthase construct, ZXA accumulated 17.35 mg/L FOH and 87.18 mg/L squalene over the same time period. Interestingly, the ZXB yeast line accumulated 70.24 mg/L FOH and almost 250 mg/L squalene.

Discussion

Terpenes and terpenoids are a highly diverse class of natural products with important biological active and commercially value. Nonetheless, the high cost and low efficiency of their isolation from natural sources and the complexity of their chemical synthesis have limited the development of single entity terpene products. Several labs, including ours, have focused on utilizing and manipulating the advantages of yeast native sterol biosynthesis pathway for the production of specific classes of terpenes, like sesquiterpenes (Asadollahi et al., 2008, 2010; Nguyen et al., 2012; Paddon et al., 2013; Ro et al., 2006; Song, 2003; Takahashi et al., 2007; Westfall et al., 2012; Yuan and Ching, 2014). However, given the advantages of yeast as a fermentable organism, we desired the development of a yeast line that would have facility for the production of diverse terpenes from those having a 10-carbon backbone (monoterpenes) to those with 30 carbon backbones (triterpenes), as well as the capacity for biochemical diversification (Takahashi et al., 2007). In the current work, we presented a methodological approach using genetic and molecular genetic tools to select yeast lines, including ZX 178-08, ZXA and ZXB that offer many of these advantages.

Several of the tools we have used in this study have been used before. For instance, nystatin has been used for more than three

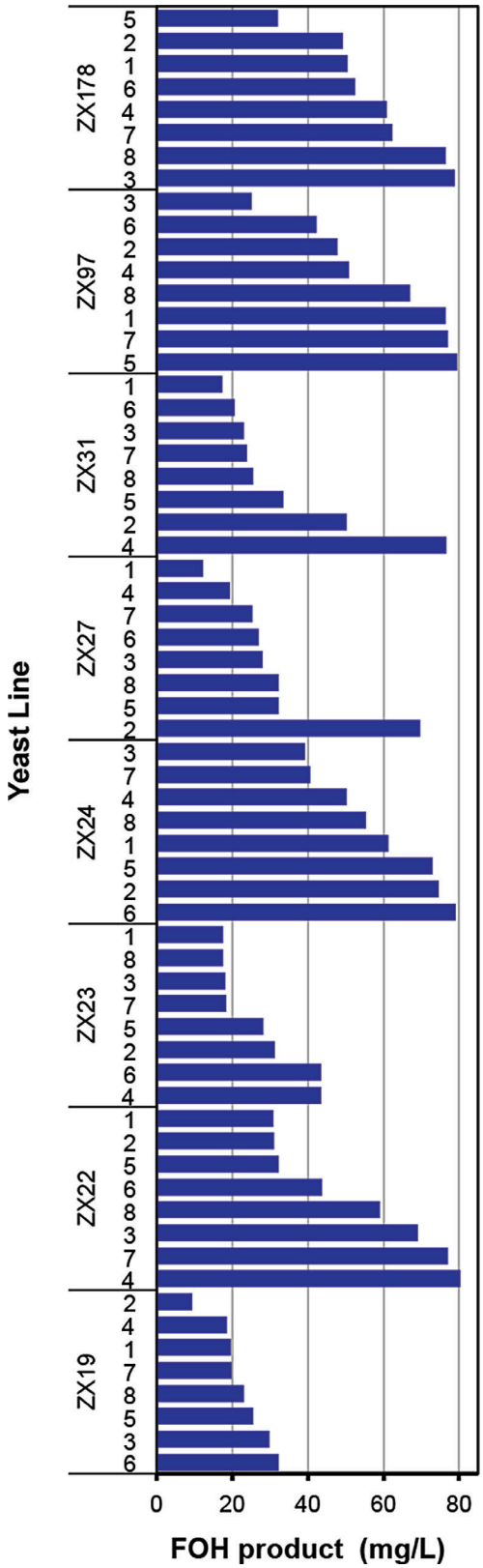

Figure 5. Quantitation of FOH levels in *SUE*, *erg*9 mutant lines of yeast demonstrated to have an exogenous sterol requirement for growth and resistance to hygromycin. Cultures were grown for 6 days prior to extracting and quantifying their farnesol (FOH) levels by GC-MS.

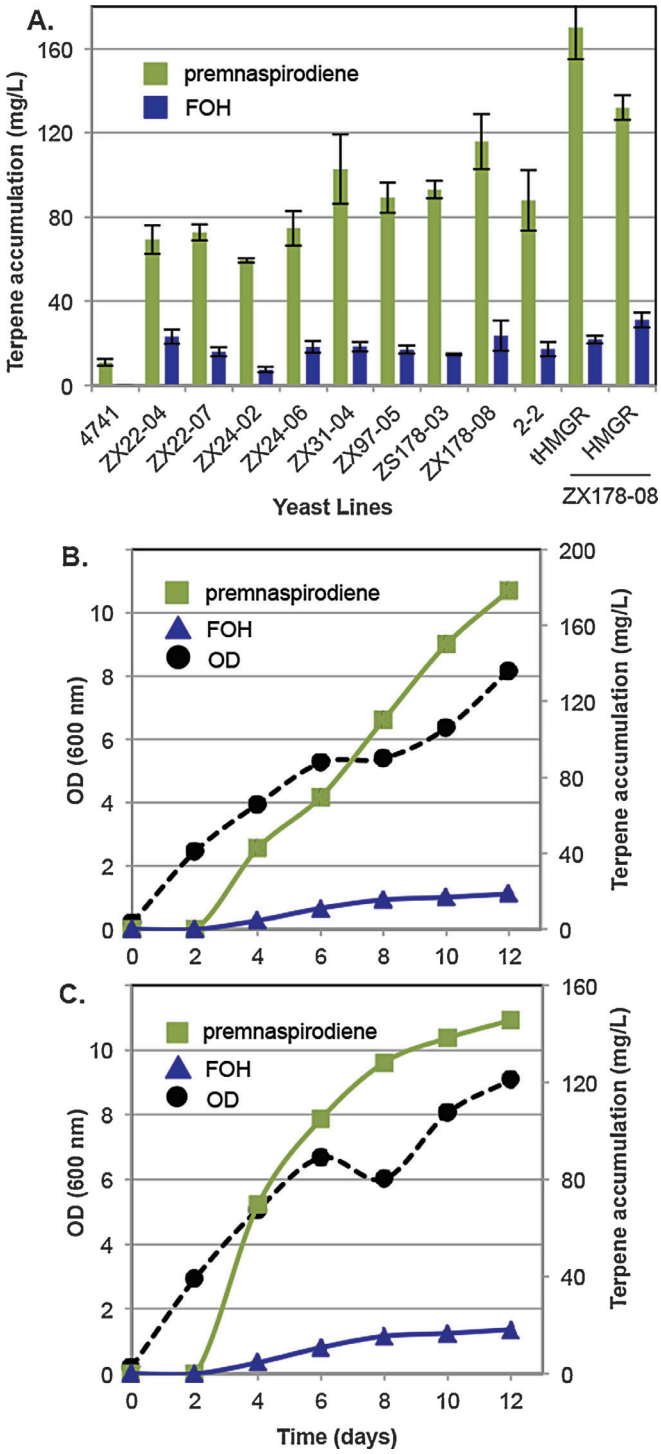

Figure 6. Comparison of terpene accumulation levels in yeast lines (ZX and 2–2 series) developed as terpene production platforms. Each of the ZX cell lines, as well as the parental line (BY4741), were independently transformed with an expression vector harboring the *Hyoscyamus* premnaspirodiene synthase gene or along with vectors harboring either a truncated (t*HMGR*) or full length hamster *HMGR* (*HMGR*). The yeast lines were then grown 12 days prior to chemically profiling for their cell constituents by GC-MS and quantifying the levels of premnaspirodiene and farnesol found in each (A). Comparison of cell culture growth, premnaspirodiene and farnesol accumulation by yeast strain ZX178-08 also co-expressing the truncated *HMGR* (B) or full length *HMGR* (C) over 12 days were also monitored.

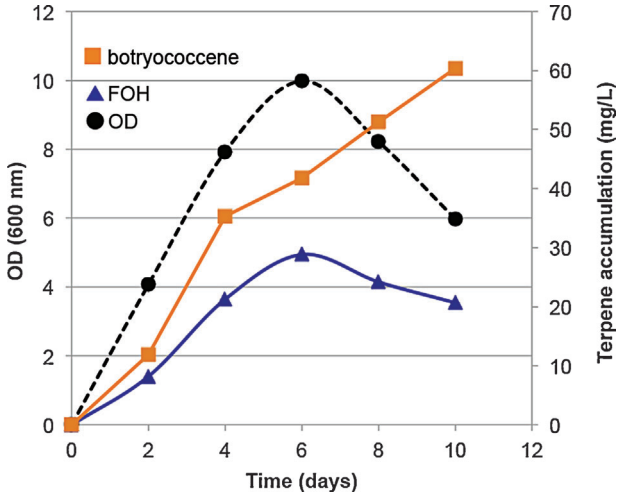

Figure 7. Comparison of cell culture growth (circles), farnesol (triangles) and botryococcene (squares) accumulation by yeast line ZX178-08 expressing a chimeric *SSL-1,3* gene encoding for botryococcene synthase under the control of an *ADH1* promoter. Farnesol and botryococcene were determined by GC-MS.

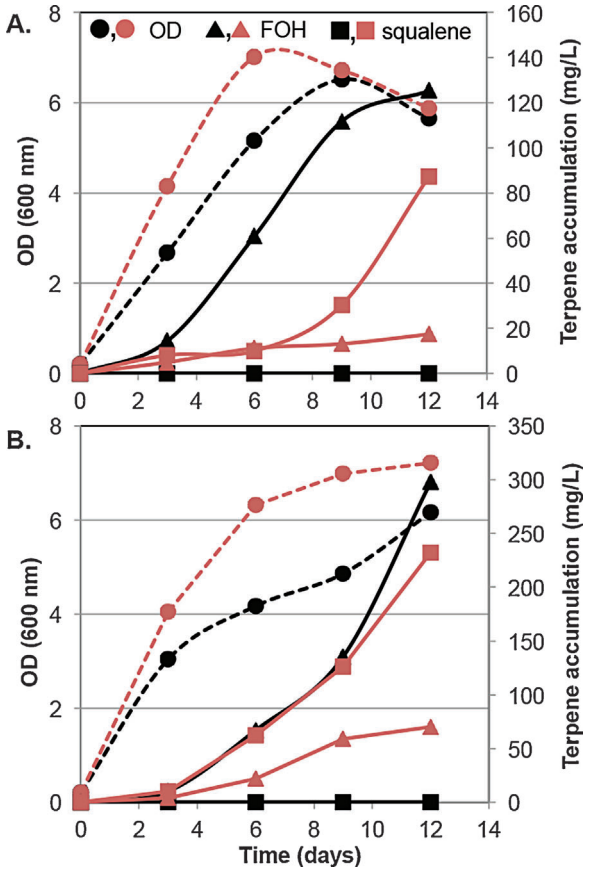

Figure 8. Comparison of cell culture growth, farnesol and squalene accumulation in yeast lines ZXA (panel A) and ZXB (panel B). Control cultures of ZXA and ZXB (black symbols) were monitored relative to lines ZXA and ZXB transformed with an expression cassette harboring an algal squalene synthase gene direct by the strong, constitutive *GPD* promoter. ZXA is derived from ZX178-08 by having an insertional deletion of the native squalene epoxidase gene (*ERG1*), while ZXB is likewise constructed except the substitution insertion also harbors an additional copy of a hamster truncated *HMGR* gene.

decades for the isolation of genes associated with ergosterol biosynthesis (Parks et al., 1995). The major difference when it was first used was that the yeast mutants had to be selected under anaerobic conditions. This was because wild type yeast could only take up exogenous sterols under anaerobic conditions. The discovery of mutants allowing for sterol uptake aerobically opened up additional opportunities for using nystatin selection. The mutant yeast genes responsible for sterol uptake enhancements have subsequently been described (Zavrel et al., 2013), and are associated with biochemical processes distantly related to the mevalonate or ergosterol biosynthetic pathway. However, Ro et al. (2006) were one of the first to recognize the advantages of these genes for assembling a yeast line capable of accumulating high levels of the amorphadiene, the sesquiterpene hydrocarbon precursor to the potent anti-malaria therapy artemisinin, to about one-half the level reported here. Westfall et al. (2012) followed up with a complementary effort examining the benefit of over-expressing each of the mevalonate biosynthetic pathway genes using chromosomal integration techniques rather than plasmid-based efforts, which with process engineering advances was capable of yielding 10's of grams of amorphadiene per liter (Paddon et al., 2013). In contrast, Asadollahi et al. (2008, 2010) created yeast lines for terpene production based on fine-tuning expression of various mevalonate biosynthetic genes using a variety of molecular genetic tools with the aim of not having to supply the cultures with exogenous sterols. Ozaydin et al. (2013) screened a yeast deletion collection for improved terpene production strain by color screening based on carotenoid production, then tested the highest carotenoid producing strains for bisabolene production. Deletion of three genes with additional mevalonate pathway modifications, improved bisabolene production from 40 mg/L to 800 mg/L in shake flask scale culture, and further to 5.2 g/L in optimized bioreactor conditions. While the levels of sesquiterpene hydrocarbons produced by these lines of yeast were comparable to others, savings in cost for medium components when scaling such a production platform were evident.

Chemical screens, especially for farnesol, have also been described for the isolation of mutants having uncontrolled flux of carbon down the mevalonate pathway (Song, 2003). However, what has not been described previously is an attempt to isolate mutants capable of taking up and complementing their sterols needs with exogenous sterols under aerobic conditions while simultaneously selecting for a dispensable mevalonate pathway using the exquisitely specific squalene synthase inhibitor squalestatin (Bergstrom et al., 1995). Our rationale, which is a unique, unbiased random mutagenesis and screening approach, did not require *a priori* hypotheses or a computational network algorithm to select what genes to manipulate. Those strategies, while successful as noted above, essentially represent trial and error testing approaches. Instead, our method relied on a positive physiological growth selection of the desired mutant phenotypes. Hence, we successfully isolated many suitable yeast lines, but

ZX178-08 possessed the best attributes for reproducible and robust production of specific terpene compounds. The other yeast lines isolated during this effort certainly possess other unknown mutations which could serve as additional resources to study the fundamental mechanisms regulating terpene and sterol biosynthesis in yeast.

In phase I, chemical mutagenesis was used to isolate mutants capable of taking up exogenously supplied sterol from medium under aerobic condition in the presence of squalestatin. In phase II, high farnesol accumulating lines were identified by GS-MS profiling when the mutants were grown under conditions mimicking the knockout of squalene synthase enzyme activity. Because farnesol is the dephosphorylated form of FPP, these high FOH accumulating lines demonstrated an enhanced carbon flux to FPP. Development of the *erg9* (yeast squalene synthase) knockout mutants in phase III relieved the need for squalestatin use. The obtained yeast lines are thus blocked in the flow of FPP to the ergosterol biosynthesis pathway, which makes for appreciable flux of carbon through all the key isoprenoid intermediates leading up to and including FPP, any of which might be diverted for the biosynthesis of any other class of terpene. In contrast, Ro et al. (2006) and Asadollahi et al. (2008) utilized an alternative strategy to control the flux of FPP to downstream sterol biosynthesis by introducing a regulatory promoter in front of the *ERG9* gene, thus attenuating the flow of FPP to sterol biosynthesis and availing FPP for the biosynthesis of other designer terpenes upon induction conditions.

Heterologous expression of the *HPS* sesquiterpene synthase gene was used to demonstrate the sesquiterpene production capability of the new developed yeast lines. In terms of overall culture growth and sesquiterpene accumulation, ZX178-08 grew about 2-times more robustly based on growth rate (time to reach cell culture saturation) and accumulated about twice the level of premnaspirodiene as previously described for the SW-24 and CALI-7 yeast lines (Takahashi et al., 2007). Over-expression of truncated and full-length *HMGR* genes encoding for the catalytic domain of HMG-CoA reductase also augmented premnaspirodiene accumulation 20–50% in shake flask experiments, comparable to the observations of Ro et al. (2006) for amorphadiene production.

Additional utility of the yeast lines developed here was provided by their assessment for triterpene production. While the accumulation of botryococcene was greater than 65 mg/L, squalene production was much more significant. This was particular so for the line ZXB, which harbors a truncated HMGR gene, inserted into the squalene epoxidase locus (*ERG*1). However, more interesting is the comparison of squalene accumulation in line ZXB harboring the algal squalene synthase gene versus FOH accumulation in ZXB without an added triterpene synthase (Fig. 8B). Farnesol accumulation in the latter case was in excess of 300 mg/L, while squalene accumulation in the former case was roughly comparable at approximately 250 mg/L. Because each squalene molecule is derived from the biosynthetic coupling of two FPP molecules, the production of squalene in ZXB is roughly equivalent to the biosynthesis of 500 mg/L of FPP, about double the amount of FOH that accumulates in ZXB without the introduced squalene synthase expression cassette. Hence, the data in Figure 8B supports the notion that more efficient utilization of FPP can result in additional carbon flux down the MVA pathway to compensate for the draw of FPP to divergent biosynthetic products, also consistent with FPP possibly playing a role in a feedback regulatory mechanism (Gardner and Hampton, 1999).

Altogether, the new yeast strains described here arise from a combination of genetic and molecular genetic screens impacting known and unknown genes resulting in alternative production platforms suitable for sesquiterpene and triterpene production, and portends well for other classes of terpene products as well. Finally, the suite of yeast lines developed here provides the resources for a genome wide association study, a study that could identify new loci contributing to metabolic fluxes and regulatory networks in an unbiased, yet directed approach (Oud et al., 2012).

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References

Ajikumar PK, Xiao WH, Tyo KEJ, Wang Y, Simeon F, Leonard E, Mucha O, Phon TH, Pfeifer B, Stephanopoulos G. 2010. Isoprenoid pathway optimization for taxol precursor overproduction in escherichia coli. Science 330(6000):70–74.

Asadollahi MA, Maury J, Moller K, Nielsen KF, Schalk M, Clark A, Nielsen J. 2008. Production of plant sesquiterpenes in *Saccharomyces cerevisiae*: Effect of ERG9 repression on sesquiterpene biosynthesis. Biotechnol Bioeng 99(3):666–677.

Asadollahi MA, Maury J, Schalk M, Clark A, Nielsen J. 2010. Enhancement of farnesyl diphosphate pool as direct precursor of sesquiterpenes through metabolic engineering of the mevalonate pathway in *Saccharomyces cerevisiae*. Biotechnol Bioeng 106(1):86–96.

Bedoukian P. 1983. Perfumery and Flavour Materials, Perfumery and Flavoring Materials, Allured Publ Corp, Carol Stream, IL.

Bergstrom JD, Dufresne C, Bills GF, Nallinomstead M, Byrne K. 1995. Discovery, biosynthesis, and mechanism of action of the zaragozic acids—potent inhibitors of squalene synthase. Ann Rev Microbiol 49:607–639.

Besumbes O, Sauret-Gueto S, Phillips MA, Imperial S, Rodriguez-Concepcion M, Boronat A. 2004. Metabolic engineering of isoprenoid biosynthesis in Arabidopsis for the production of taxadiene, the first committed precursor of Taxol. Biotechnol Bioeng 88(2):168–175.

Bhilwade HN, Tatewaki N, Nishida H, Konishi T. 2010. Squalene as novel food factor. Current Pharm Biotechnol 11(8):875–880.

Boghigian BA, Armando J, Salas D, Pfeifer BA. 2012. Computational identification of gene over-expression targets for metabolic engineering of taxadiene production. Appl Microbiol Biotechnol 93(5):2063–2073.

Bourot S, Karst F. 1995. Isolation and characterization of the *saccharomyces cerevisiae* sut1 gene involved in sterol uptake. Gene 165(1):97–102.

Buckingham J. 2003. Dictionary of natural products. London: Chapman & Hall (CD rom).

Casida JE. 2009. Pest toxicology: The primary mechanisms of pesticide action. Chem Res Toxicol 22(4):609–619.

Chappell J, Wolf F, Proulx J, Cuellar R, Saunders C. 1995. Is the reaction catalyzed by 3-hydroxy-3-methylglutaryl coenzyme-a reductase a rate-limiting step for isoprenoid biosynthesis in plants. Plant Physiol 109(4):1337–1343.

Chin DJ, Gil G, Faust JR, Goldstein JL, Brown MS, Luskey KL. 1985. Sterols accelerate degradation of hamster 3-hydroxy-3-methylglutaryl coenzyme A reductase encoded by a constitutively expressed cDNA. Mol Cell Biol 5(4):634–641.

Engels B, Dahm P, Jennewein S. 2008. Metabolic engineering of taxadiene biosynthesis in yeast as a first step towards Taxol (Paclitaxel) production. Metabolic Engin 10(3-4):201–206.

Faulkner A, Chen X, Rush J, Horazdovsky B, Waechter CJ, Carman GM, Sternweis PC. 1999. The LPP1 and DPP1 gene products account for most of the isoprenoid phosphate phosphatase activities in Saccharomyces cerevisiae. J Biol Chem 274(21):14831–14837.

Fischer MJC, Meyer S, Claudel P, Bergdoll M, Karst F. 2011. Metabolic engineering of monoterpene synthesis in yeast. Biotechnol Bioeng 108(8):1883–1892.

Gardner RG, Hampton RY. 1999. A highly conserved signal controls degradation of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase in eukaryotes. J Biol Chem 274(44):31671–31678.

Gil G, Faust JR, Chin DJ, Goldstein JL, Brown MS. 1985. Membrane-bound domain of HMG CoA reductase is required for sterol-enhanced degradation of the enzyme. Cell 41(1):249–258.

Hillen LW, Pollard G, Wake LV, White N. 1982. Hydrocracking of the oils of *Botryococcus braunii* to transport fuels. Biotechnol Bioeng 24(1):193–205.

Huang ZR, Lin YK, Fang JY. 2009. Biological and pharmacological activities of squalene and related compounds: Potential uses in cosmetic dermatology. Molecules 14(1):540–554.

Janke C, Magiera MM, Rathfelder N, Taxis C, Reber S, Maekawa H, Moreno-Borchart A, Doenges G, Schwob E, Schiebel E and others. 2004. A versatile toolbox for PCR-based tagging of yeast genes: new fluorescent proteins, more markers and promoter substitution cassettes. Yeast 21(11):947–962.

Jennewein S, Croteau R. 2001. Taxol: biosynthesis, molecular genetics, and biotechnological applications. Appl Microbiol Biotechnol 57(1-2):13–19.

Keasling J. 2009. Synthetic biology in pursuit of inexpensive, effective, anti-malarial drugs. Biosocieties 4(2-3):275–282.

Kindermans JM, Pilloy J, Olliaro P, Gomes M. 2007. Ensuring sustained ACT production and reliable artemisinin supply. Malaria J 6:125.

Kirby J, Romanini DW, Paradise EM, Keasling JD. 2008. Engineering triterpene production in *Saccharomyces cerevisiae* beta-amyrin synthase from *Artemisia annua*. FEBS J 275(8):1852–1859.

Maertens JA. 2004. History of the development of azole derivatives. Clinical Microbiol Infect 10:1–10.

Malik S, Cusido RM, Mirjalili MH, Moyano E, Palazon J, Bonfill M. 2011. Production of the anticancer drug taxol in *Taxus baccata* suspension cultures: A review. Process Biochem 46(1):23–34.

Martin VJJ, Pitera DJ, Withers ST, Newman JD, Keasling JD. 2003. Engineering a mevalonate pathway in *Escherichia coli* for production of terpenoids. Nature Biotechnol 21(7):796–802.

Mathis JR, Back K, Starks C, Noel J, Poulter CD, Chappell J. 1997. Pre-steady-state study of recombinant sesquiterpene cyclases. Biochemistry 36(27): 8340–8348.

Nguyen TD, MacNevin G, Ro DK. 2012. De novo synthesis of high-value plant sesquiterpenoids in yeast. Methods Enzymol 517:261–278.

Nicolaou KC, Yang Z, Liu JJ, Ueno H, Nantermet PG, Guy RK, Claiborne CF, Renaud J, Couladouros EA, Paulvannan K and others. 1994. Total synthesis of taxol. Nature 367(6464):630–634.

Niehaus TD, Okada S, Devarenne TP, Watt DS, Sviripa V, Chappell J. 2011. Identification of unique mechanisms for triterpene biosynthesis in Botryococcus braunii. Proc Natl Acad Sci U S A 108(30):12260–12265.

Okada S, Devarenne TP, Chappell J. 2000. Molecular characterization of squalene synthase from the green microalga *Botryococcus braunii*, race B. Arch Biochem Biophys 373(2)):307–317.

Okada S, Murakami M, Yamaguchi K. 1995. Hydrocarbon composition of newly isolated strains of the green microalga *Botryococcus braunii*. J Appl Phycol 7:555–559.

Oud B, van Maris AJ, Daran JM, Pronk JT. 2012. Genome-wide analytical approaches for reverse metabolic engineering of industrially relevant phenotypes in yeast. FEMS Yeast Res 12(2):183–196.

Özaydin B, Burd H, Lee TS, Keasling JD. 2013. Carotenoid-based phenotypic screen of the yeast deletion collection reveals new genes with roles in isoprenoid production. Metab Eng 15:174–183.

Paddon CJ, Westfall PJ, Pitera DJ, Benjamin K, Fisher K, McPhee D, Leavell MD, Tai A, Main A, Eng D and others. 2013. High-level semi-synthetic production of the potent antimalarial artemisinin. Nature 496:528–532.

Parks LW, Smith SJ, Crowley JH. 1995. Biochemical and physiological effects of sterol alterations in yeast-a review. Lipids 30(3):227–230.

Ramya HG, Palanimuthu V, Singla R. 2013. An introduction to patchouli (Pogostemon cablin Benth.) —A medicinal and aromatic plant: It's importance to mankind. Agric Eng Int: CIGR 15:243–250.

Reddy LH, Couvreur P. 2009. Squalene: A natural triterpene for use in disease management and therapy. Advanced Drug Delivery Rev 61(15):1412–1426.

Ro DK, Paradise EM, Ouellet M, Fisher KJ, Newman KL, Ndungu JM, Ho KA, Eachus RA, Ham TS, Kirby J and others. 2006. Production of the antimalarial drug precursor artemisinic acid in engineered yeast. Nature 440(7086):940–943.

Seki H, Ohyama K, Sawai S, Mizutani M, Ohnishi T, Sudo H, Akashi T, Aoki T, Saito K, Muranaka T. 2008. Licorice beta-amyrin 11-oxidase, a cytochrome P450 with a key role in the biosynthesis of the triterpene sweetener glycyrrhizin. Proc Natl Acad Sci U S A 105(37):14204–14209.

Shianna KV, Dotson WD, Tove S, Parks LW. 2001. Identification of a UPC2 homolog in *Saccharomyces cerevisiae* and its involvement in aerobic sterol uptake. J Bacteriol 183(3):830–834.

Silva L, Coutinho A, Fedorov A, Prieto M. 2006. Competitive binding of cholesterol and ergosterol to the polyene antibiotic nystatin. A fluorescence study. Biophys J 90(10):3625–3631.

Siperstein MD, Guest MJ. 1960. Studies on the site of the feedback control of cholesterol synthesis. J Clin Invest 39:642–652.

Song LS. 2003. Detection of farnesyl diphosphate accumulation in yeast ERG9 mutants. Anal Biochem 317(2):180–185.

Takahashi S, Yeo Y, Greenhagen BT, McMullin T, Song L, Maurina-Brunker J, Rosson R, Noel JP, Chappell J. 2007. Metabolic engineering of sesquiterpene metabolism in yeast. Biotechnol Bioeng 97(1):170–181.

Tu YY. 2011. The discovery of artemisinin (qinghaosu) and gifts from Chinese medicine. Nature Med 17(10):1217–1220.

Wall ME, Wani MC. 1995. Paclitaxel—from discovery to clinic. In: Georg GI, Chem TT, Ojima I, Vyas DM, editors. Taxane anticancer agents: Basic science and current status. ACS Symposium Series, Washington, D.C., p 18–30.

Westfall PJ, Pitera DJ, Lenihan JR, Eng D, Woolard FX, Regentin R, Horning T, Tsuruta H, Melis DJ, Owens A and others. 2012. Production of amorphadiene in yeast, and its conversion to dihydroartemisinic acid, precursor to the antimalarial agent artemisinin. Proc Natl Acad Sci USA 109(3):111–118.

Wu SQ, Schalk M, Clark A, Miles RB, Coates R, Chappell J. 2006. Redirection of cytosolic or plastidic isoprenoid precursors elevates terpene production in plants. Nature Biotechnol 24(11):1441–1447.

Yared JA, Tkaczuk KHR. 2012. Update on taxane development: New analogs and new formulations. Drug Des Dev Ther 6:371–384.

Yuan J, Ching CB. 2014. Combinatorial engineering of mevalonate pathway for improved amorpha-4, 11-diene production in budding yeast. Biotechnol Bioeng 111(3):608–617.

Zavrel M, Hoot SJ, White TC. 2013. Comparison of sterol import under aerobic and anaerobic conditions in three fungal species, *Candida albicans*, *Candida glabrata*, and *Saccharomyces cerevisiae*. Eukaryot Cell 12(5):725–738.

Zhang DL, Jennings SM, Robinson GW, Poulter CD. 1993. Yeast squalene synthase—expression, purification, and characterization of soluble recombinant enzyme. Arch Biochem Biophys 304(1):133–143.

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