

Metabolic Engineering of Sesquiterpene Metabolism in Yeast

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ABSTRACT: Terpenes are structurally diverse compounds that are of interest because of their biological activities and industrial value. These compounds consist of chirally rich hydrocarbon backbones derived from terpene synthases, which are subsequently decorated with hydroxyl substituents catalyzed by terpene hydroxylases. Availability of these compounds is, however, limited by intractable synthetic means and because they are produced in low amounts and as complex mixtures by natural sources. We engineered yeast for sesquiterpene accumulation by introducing genetic modifications that enable the yeast to accumulate high levels of the key intermediate farnesyl diphosphate (FPP). Co-expression of terpene synthase genes diverted the enlarged FPP pool to greater than 80 mg/L of sesquiterpene. Efficient coupling of terpene production with hydroxylation was also demonstrated by coordinate expression of terpene hydroxylase activity, yielding 50 mg/L each of hydrocarbon and hydroxylated products. These yeast now provide a convenient format for investigating catalytic coupling between terpene synthases and hydroxylases, as well as a platform for the industrial production of high value, single-entity and stereochemically unique terpenes.

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Introduction

Terpenes are one of the largest and most diverse families of chemical compounds found in nature. For instance, thousands of C10 monoterpenes, C15 sesquiterpenes, and C20 diterpenes have been identified, and these represent just three subfamilies within a group of seven members, albeit 3 of the largest (Buckingham, 2003; Dewick, 2002). Equally important, the already identified chemical diversity is estimated to represent only a small fraction, 1–10%, of the natural variability. The structural diversity arises in several different ways. First, the biosynthetic precursors for mono-, sesqui-, and di-terpenes are high energy intermediates that undergo initial transformations catalyzed by enzymes referred to as synthases, yielding highly complex linear and cyclic carbon scaffolds (Davis and Croteau, 2000). The precursors, geranyl diphosphate (GPP) for monoterpenes, farnesyl diphosphate (FPP) for sesquiterpenes, and geranylgeranyl diphosphate (GGPP) for diterpenes, consist of branched, five carbon repeating units terminated by a diphosphate substituent. Cleavage of the diphosphate yields

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a reactive linear carbocation intermediate that can, for instance, undergo sequential rounds of cyclization, double bond, methyl and methylene migrations, and neutralization by either proton abstraction or quenching by water capture. The net result is often multiple ring systems with several chiral centers and stereochemical positioning of methyl and isopropenyl substituents. A second layer of structural diversity for terpenes is imposed by subsequent modifying reactions of the terpene scaffold, such as single to multiple regio- and stereo-specific hydroxylation (Lupien et al., 1999; Takahashi et al., 2005), lactonization (de Kraker et al., 2002), epoxidation (Helvig et al., 2004), oxidations and reductions (Davis et al., 2005; Ringer et al., 2005), halogenation (Polzin and Rorrer, 2003), acetylations (Menhard and Zenk, 1999; Walker et al., 1999), and glycosylations (Richman et al., 2005). These later modifications appear particularly important for imparting chemical properties that correlate with biological activities of the final terpene products.

Identification and structural elucidation of terpenes is frequently a difficult and arduous task because these compounds are often in very low abundance in their natural sources and components of complex mixtures, including stereo- and regio-isomers. Hence, advances in extraction methodologies (Peres et al., 2006) as well as analytical analyses (Fraga, 2005) have benefited these efforts tremendously. However, an alternative means towards uncovering the breadth of this diversity is also apparent from recent advances in the isolation and characterization of the genes encoding for these biosynthetic systems. Functional identification of the terpene biosynthetic genes necessarily entails expression of the corresponding gene and most routinely an *in vitro* characterization of the isolated enzyme (i.e., Wu et al., 2005). Mechanisms for *in vivo* assessment have also been described including the use of knockout mutations and RNA suppression technologies and the observed loss of a particular metabolite profile (Tholl et al., 2005; Wang and Wagner, 2003). Overexpression of a gene of interest in either a prokaryotic or eukaryotic expression host has also been exploited. For example, genes for triterpene metabolism have been characterized by their overexpression in yeast, which have an endogenous supply of farnesyl diphosphate (FPP), a necessary precursor, and an internal membrane system necessary for proper expression of these integral membrane bound enzymes and sites for end-product accumulation (Fazio et al., 2004; Shibuya et al., 2006).

Several groups have reported important progress in developing bacterial systems for terpene biosynthesis by engineering unique metabolic pathways or adding extra copies of genes for putative rate-limiting steps into the native biosynthetic machinery. Martin et al. (2003) observed significant accumulation of the sesquiterpene hydrocarbon amorphadiene in *E. coli* engineered with early steps of the eukaryotic mevalonate pathway and the plant amorphadiene synthase gene. Reiling et al. (2004) demonstrated that upregulation of putative limiting steps of the endogenous prokaryotic methyl erythritol phosphate pathway along

with additional prenyl transferase activities for GPP and GGPP biosynthesis, key branch point intermediates, were sufficient to improve mono- and di-terpene hydrocarbon accumulation in *E. coli*. Carter et al. (2003) had likewise reported improved monoterpene accumulation in *E. coli* cultures by engineering GPP biosynthesis coupled with limonene synthase using plant prenyl transferase and monoterpene synthase genes. However, co-transformation with genes coding for limonene hydroxylase, cytochrome P450 reductase (necessary to provide reducing equivalents to the hydroxylase enzyme) and carveol dehydrogenase did not support further *in vivo* modification of the limonene scaffold. As noted by these investigators, the inability of these downstream enzymes to couple with the early biosynthetic steps could result from structural limitations (not properly assembled and associated with an endomembrane system as in eukaryotes) or a missing or limiting co-factor (i.e., NADPH levels). Hence, while bacterial systems may be suitable for the initial biosynthetic steps for terpene hydrocarbons, these bacterial platforms are missing capacities for downstream processing/modifying enzymes.

Yeast platforms offer many of the same advantages that prokaryotic systems do for terpene hydrocarbon production, plus may provide the biosynthetic machinery necessary for the proper functioning of the downstream modifying enzymes like cytochrome P450 hydroxylases. Jackson et al. (2003) reported that the best yields of the sesquiterpene epi-cedrol, up to 370 µg/L, resulted from overexpression of the corresponding terpene synthase gene and a truncated form of 3-hydroxy-3-methylglutaryl Coenzyme A reductase gene in the presence of a mutation conferring sterol uptake (*upc2-1*) from the exogenous media. Overexpression of a native FPP synthase gene, however, did not improve sesquiterpene accumulation in these yeast lines. In contrast, DeJong et al. (2006) observed that overexpression of a GGPP synthase gene in combination with the taxadiene synthase gene did result in accumulation of 0.7–1 mg/L of the taxadiene hydrocarbon. Similar to those results reported by Carter et al. (2003) using *E. coli*, no further hydroxylation of the diterpene scaffold was observed when any one of several P450 taxoid hydroxylases genes were co-expressed in the yeast. This metabolic restriction, the authors noted, may arise because of poor expression of the corresponding P450 genes or the limited coupling and supply of reducing equivalents between the endogenous yeast NADPH-cytochrome P450 reductase and the introduced plant P450 hydroxylases. Very recently, Ro et al. (2006) reported that sesquiterpene production in yeast could be dramatically improved with a combination of alterations including downregulation of squalene synthase (*ERG9*), introduction of the *upc2-1* mutation (which upregulates a global transcription activity), overexpression of FPP synthase and a catalytic form of 3-hydroxy-3-methylglutaryl CoA reductase (HMGR) in combination with inducible expression of a amorphadiene synthase, a sesquiterpene synthase. These investigators also demonstrated that co-expression of a sesquiterpene hydroxylase (cytochrome P450) gene and

cytochrome P450 reductase gene were sufficient to generate artemisinic acid, a multi-hydroxylated sesquiterpene.

The current work was also undertaken to develop yeast as a production platform for terpenes and, in particular, to capture the full chemical diversity and potential of sesquiterpene biosynthesis in this heterologous expression host (Fig. 1). We describe here the utilization of yeast strains engineered to enhance carbon flux through the mevalonate pathway and accumulate high intracellular levels of farnesyl diphosphate (FPP), a key intermediate in sesquiterpene biosynthesis; the diversion of this intermediate for high-level production of diverse sesquiterpene hydrocarbons in lines engineered with terpene synthase genes; and finally the functional hydroxylation of a sesquiterpene scaffold by co-expression of a cognate cytochrome P450 hydroxylase and cytochrome P450 reductase. Such developments support long range objectives to facilitate functional characterization of putative terpene biosynthetic genes identified in genomic sequencing efforts (Fazio et al., 2004; Shibuya et al., 2006; Wu et al., 2005), to generate large quantities of end-product terpenes sufficient for detailed chemical analyses and diverse biological and industrial testing, as well as to combine tools for molecular evolution of terpene biosynthetic enzymes (Greenhagen, 2003; Greenhagen et al., 2006; Yoshikuni et al.,

2006) with a production platform suitable for the molecular dissection of catalytic activities and the discovery of novel terpene compounds.

Materials and Methods

Materials and Media

All chemical reagents were obtained from Sigma-Aldrich (St. Louis, MO), Fluka (Buchs, Switzerland), or Fisher Scientific (Chicago, IL), while reagents for molecular manipulations were from Stratagene (San Diego, CA) or Invitrogen (Carlsbad, CA).

Bacteria and yeast were grown using standard culture practices. SGI media for selection of WAT11 after transformation consisted of 0.1% Bacto-casamino acids, 0.7% yeast nitrogen base, and 2% glucose. YPSE media for expression of tobacco epi-aristolochene synthase (TEAS) gene under *GAL10* promoter consisted of 1% Bacto-yeast extract, 1% Bacto-peptone, 0.5% sucrose, and 3% ethanol. YPD media for growing yeast without selection consisted of 1% Bacto-yeast extract, 2% Bacto-peptone, and 2% glucose (or 0.5% glucose for select experiments), and was supplemented with ergosterol (5 mg/L) where indicated (YPDE media). Minimal media, SCE (pH 5.5), contained 0.67% Bacto-yeast nitrogen base (without amino acids), 2% dextrose, 0.6% succinic acid, uracil (20 mg/L), L-tryptophan (20 mg/L), L-histidine (20 mg/L), L-arginine (20 mg/L), L-methionine (20 mg/L), L-tyrosine (30 mg/L), L-leucine (30 mg/L), L-isoleucine (30 mg/L), L-lysine (30 mg/L), L-phenylalanine (50 mg/L), L-glutamic acid (100 mg/L), L-aspartic acid (100 mg/L), L-valine (150 mg/L), L-threonine (200 mg/L), L-serine (400 mg/L), and 40 mg/L ergosterol from a stock of 10 mg/mL in 50% Triton X-100, 50% ethanol. (In separate experiments, ergosterol and Triton X-100 concentrations were optimized for maximum growth of the yeast cell cultures and terpene hydrocarbon production.) Selection media was prepared similarly except without supplementing the media with the indicated reagent.

Yeast Strains

The WAT11 yeast strain was previously engineered with an Arabidopsis NADPH-cytochrome P450 reductase gene (Urban et al., 1997) and kindly provided by P. Urban (Centre de Génétique Moléculaire, CNRS, Gif-sur-Yvette, France (see Supplementary Table I).

The SW24, CALI5-1, and CALI7-1 yeast strains were developed by researchers at Bio-Technical Resources (Manitowoc, WI) as detailed in US patent 6,531,303, and were derived from wild-type strain ATCC 28383 (*MATa*) (see Supplementary Table I). Mutagenesis of ATCC 28383 with nitrous acid followed by selection for growth in the presence of nystatin and exogenous cholesterol yielded a strain having an *erg9* mutation (single base pair deletion) as

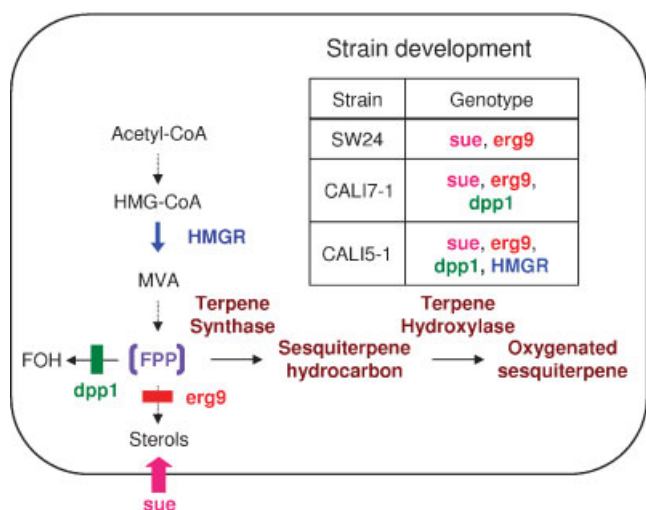


Figure 1. The mevalonate (MVA) pathway and those steps engineered in the current study for novel sesquiterpene production in yeast. The MVA pathway is localized to the cytoplasm in eukaryotic cells and supports the biosynthesis of numerous terpene macromolecules. In *Saccharomyces cerevisiae*, ergosterol is the dominant terpene derived from FPP, a highly regulated 15-carbon intermediate in the MVA pathway (Gardner and Hampton, 1999). Various combinations of mutations in genes coding for a phosphatase (*DPP1*) and squalene synthase (*ERG9*), along with selection for aerobic uptake of exogenous ergosterol (*sue*) and engineering of a soluble, unregulated form of 3-hydroxy-3-methylglutaryl CoA reductase (*HMGR*) should yield yeast strains capable of accumulating excess levels of FPP. The potential to divert the excess FPP to novel sesquiterpene hydrocarbon biosynthesis was assessed by introducing key branch point enzymes (terpene synthases), and the potential to further modify these hydrocarbon skeletons was determined by co-transformation with a gene encoding for a cytochrome P450 hydroxylating enzyme (terpene hydroxylase).

well as an uncharacterized mutation supporting aerobic sterol uptake enhancement (*sue*). An additional round of chemical mutagenesis of the *erg9* mutant with EMS and selection for 5-fluoroorotic acid resistant cells allowed for the isolation of a strain auxotrophic for uracil due to a mutation in the *URA3* gene. This strain was genetically altered to contain a deletion in the *HIS3* gene using a gene transplacement plasmid as described by Sikorski and Hieter (1989) with the pop-in/pop-out gene replacement procedure developed by Rothstein (1991). The *his3* mutant was named SWE23- Δ H1, which was further modified to contain mutations in the *leu2* and *trp1* genes using gene transplacement plasmids (Sikorski and Hieter, 1989) with the pop-in/pop-out gene replacement procedure (Rothstein, 1991) as described above. One of the resulting strains containing *erg9*, *ura3*, *his3*, *leu2*, *trp1*, and *sue* mutations was further modified by exchanging the original *erg9* frameshift mutation with the *erg9 Δ ::HIS3* allele. The resulting strain is referred to as SW23B, and has the following genotype: *ura3*, *leu2*, *trp1*, *his3*, *erg9::HIS3*, *sue*. This strain was further modified by replacing the original *ura3* mutation with a *ura3 Δ* allele, resulting in strain SW24.

In order to obtain strains that overexpressed HMGR, copies of the gene coding for the catalytic domain of HMG2 were integrated into the genome of strain SW23B. This was accomplished by first constructing a plasmid that allowed for multiple integrations of the *HMG2* gene into the spacer region of the rRNA genes. This approach was modeled after the rDNA integration method described by Lopes et al. (1989). A DNA fragment derived from pRH124-31 which contained the *HMG2* expression construct was combined with a poorly expressed *TRP1* gene. The truncated *HMG2* gene was flanked by the *GPD* promoter (Mumberg et al., 1995) and a *PGK* termination sequence (Perkins et al., 1983). These genes were then flanked on both ends by sequences derived from the non-transcribed spacer region between the 35S and 5S rRNA genes. The *TRP1* promoter was partially defective in order to reduce expression of the *TRP1* gene, and thereby provide a means of selection for high copy number integration of the final expression cassette. The DNA fragment containing the *HMG2/TRP1/rDNA* gene fusions was transformed into strain SW23B, and strain SW23B#74 identified as carrying eight copies of the integration construct.

CALI7-1 (*leu2*, *trp1*, *his3*, *ura3 Δ* , *erg9 Δ ::HIS3*, *sue*, *dpp1*) was developed by transforming SW24 with a *dpp1 Δ* transplacement plasmid using the pop-in/pop-out gene replacement method described above. Likewise, CALI5-1 (*leu2*, *trp1*, *his3*, *ura3 Δ* , *erg9 Δ ::HIS3*, *sue*, *dpp1*, *HMG2cat/TRP1::rDNA*) was derived by transforming of SW23B#74 with the *dpp1 Δ* transplacement plasmid.

Molecular Manipulations

All molecular manipulations were performed according to standard methodologies described in Sambrook et al.

(1989). The TEAS (Starks et al., 1997) (GenBank accession L04680), HPS (Mathis et al., 1997) (accession U20187), and 5-epi-aristolochene dihydroxylase (EAH) (Takahashi et al., 2005) (accession AF368377) genes used in these studies have been described previously. The CVS gene was isolated using an RT-PCR strategy and mRNA isolated from grapefruit as described by Greenhagen (2003) (accession AF411120), and the tobacco cytochrome P450 reductase (P450R) gene was isolated by RT-PCR according to the information provided by Yamada et al. (1998). The correct DNA sequence for all the genes was confirmed. Supplementary Figure 1 provides a cartoon depiction of all the gene constructs developed for this work.

Yeast expression vectors pESC-TRP and pESC-LEU were obtained from Stratagene, and YEp352-URA derived from YEp352 (Hill et al., 1986) and harboring an ADH1 promoter and ADH1 terminator was obtained from Bio-Technical Resources, and these vectors were modified as illustrated in Supplementary Figure 1. YEp352-URA was modified by inserting a gateway cloning cassette (RfB) (Hartley et al., 2000) into the *SmaI* restriction site to construct YEp-GW-URA. This vector contains two *BamHI* sites (one 5' to ADH1 promoter and the other 3' to the ADH terminator). To expand availability of this vector, the former *BamHI* site was replaced with *NheI* site by site-directed mutagenesis using the Quikchange methodology (Stratagene). The resulting vector was designated as YEp-GW-URA-*NheI/BamHI*.

To generate pESC-GW-LEU, the pESC-LEU vector was digested with *NheI* and *SpeI* to eliminate the Gal promoter and multi-cloning site sequence. The ADH1 promoter-gateway cloning cassette (RfB)-ADH1 terminator fragment containing terminal *NheI* and *SpeI* sites was amplified by PCR using YEp-GW-URA as the template, and inserted into corresponding sites of pESC-LEU to create pESC-GW-LEU. The sesquiterpene synthase genes TEAS, HPS, CVS and the sesquiterpene hydroxylase gene EAH genes were cloned into the indicated destination vector (YEp-GW-URA or pESC-GW-LEU) via pDONR221-Kan vector according to the manufacturer's instructions (Invitrogen).

The YEp-P450R-EAH-URA was constructed in several steps. Because the P450R gene contains an *EcoRI* site in the middle of its open reading frame, the restriction site was knocked out by site-directed mutagenesis without changing corresponding amino acids (Quikchange protocol, Stratagene). The P450R gene was then amplified by using PCR primers harboring terminal *EcoRI* and *HindIII* sites and inserted into the corresponding sites of the YEp352-URA vector to generate YEp-P450R-URA. The ADH1 promoter-P450R-ADH terminator cassette was amplified using PCR primers containing *NheI* sites and the amplicon inserted into *NheI* site of YEp-GW-URA-*NheI/BamHI*, yielding YEp-P450R-GW-URA. YEp-P450R-EAH-URA was generated via an LR reaction between YEp-P450R-GW-URA and pDONR221-EAH according to the manufacturer's recommendations (Invitrogen).

To construct YEp-TEAS-P450R-URA, the TEAS gene was inserted into the *EcoRI/SstI* sites of the YEp352-URA vector.

As described above, a *Bam*HI site within YEp-TEAS-URA was replaced with a *Nhe*I site by site-directed mutagenesis. The resulting vector (YEp-TEAS-URA-*Nhe*I/*Bam*HI) was digested with *Bam*HI, and a ADH1 promoter-P450R-ADH terminator cassette was inserted into the corresponding site to create YEp-TEAS-P450R-URA. The YEp-EAH-TEAS-P450R-URA vector was generated by first inserting the EAH gene into the *Eco*RI/*Sst*I sites of YEp352-URA. The ADH1 promoter-EAH-ADH terminator cassette was amplified using PCR primers containing *Nhe*I sites and inserted into the corresponding site of the YEp352-URA vector to yield YEp-EAH-TEAS-P450R-URA.

Yeast Transformation and Culture Performance

Yeast strains were transformed with the respective vector constructs using the lithium acetate procedure of Gietz and Schiestl (1995), followed by selection for prototrophic growth on minimal media without supplementation for the appropriate metabolite. Five to 50 independent colonies were subsequently picked for confirmation of the recombinant vector (PCR screen) and characterized for terpene production. Small cultures of independent colonies were grown in 2–10 mL of minimal media and aliquots of the cultures analyzed for terpene production after 4 days. An equal volume of acetone was vigorously mixed with an aliquot of cell culture, then incubated at room temperature for another 10 min. Two volumes of pentane were added, shaken, and the phases separated by brief centrifugation in a clinical centrifuge. The pentane extract was analyzed directly by GC-MS, or in some cases concentrated under a N₂ stream before analysis. Independent colonies from a single transformation experiment typically exhibited 10–20% variance for terpene production between cultures, but in unique cases was as much as twofold. Cultures exhibiting the highest terpene production levels were chosen for further studies and archived as glycerol stocks at –80°C.

Selected lines were characterized for cell growth and terpene production using 50–100 mL cultures. Starter cultures grown to saturation in minimal media were inoculated at 100-fold dilutions into SCE media and 1 mL aliquots withdrawn at daily intervals for 10–15 days. Cell growth was monitored as the change in optical density at 600 nm, using appropriate dilutions for cultures at later stages of growth. Terpene production was determined similar to the initial screening method except that the initial extraction was performed with 15% ethyl acetate, 85% hexane. The organic extract was then concentrated under N₂ to approximately 10% of the original volume, and re-adjusted back to the original extraction volume with 100% hexane. The extract was then applied to a silica gel column (5 × 15 mm). The flow-through plus 2 volumes of hexane wash were collected and designated as the hydrocarbon fraction. Oxygenated terpenes were then eluted with 70% ethyl acetate, 30% hexane. When necessary, fractions were concentrated under a N₂ stream before GC-MS analysis.

Terpene quantification was performed with a Thermo Finnigan DSQ GC/MS system equipped with a Restec Rtx-5 capillary column (30 m × 0.32 mm, 0.25 μm phase thickness). Samples were injected in the splitless mode at 250°C with an initial oven temperature of 70°C for 1 min, followed by an 8°C/min gradient to 230°C, then 20°C/min to 300°C. Mass spectra were recorded at 70 eV, scanning from 35 to 300 atomic mass units, and compared to authentic standards (retention times and mass spectra) for verification (Takahashi et al., 2005). Quantification was based on total ion counts and calibrated relative to several standards, including valencene and α-cedrene (for terpene hydrocarbons), and nootkatone and capsidiol (for oxygenated terpenes). Extraction efficiencies of terpenes from the yeast cultures were calculated from the recovery of 10 μg of α-cedrene (dissolved in DMSO) added to the initial culture aliquot (1 mL), and varied from 50% (if the final sample had to be concentrated before GC-MS analysis) to greater than 85% (if no concentration were necessary).

Results

Assessment of Standard Baker's Yeast

We initially evaluated standard laboratory lines of yeast for sesquiterpene production and more specifically WAT11 (Table I). Urban et al. (1997) developed the WAT11 line for the purpose of characterizing eukaryotic P450 enzymes, enzymes that require a cytochrome P450 reductase accessory protein to supply reducing equivalents to the respective P450s, and we had used this line for the overexpression and in vitro characterization of a sesquiterpene hydroxylase previously (Takahashi et al., 2005). The TEAS gene was inserted downstream of a galactose inducible promoter (GAL10) in the commercially available pESC vector and the recombinant vector transformed into the WAT11 line. Colonies selected for tryptophan prototrophy and verified as

Table I. Engineered sesquiterpene biosynthesis in yeast strain WAT11 is gene promoter and culture media dependent.

Cell line	Promoter	Terpene synthase	Culture media	Terpene production (μg/L)
WAT11	Gal10	TEAS	YPSE	1.2
WAT11	ADH1	TEAS	YPD	230

WAT11 (Urban et al., 1997) is a standard laboratory strain of *Saccharomyces cerevisiae* that was transformed with a tryptophan prototrophic selection pESC vector harboring a galactose-inducible promoter (GAL10) fused to the sesquiterpene synthase TEAS gene, or a uracil prototrophic selection YEp352 vector harboring a constitutive ADH1 promoter fused to the TEAS gene. WAT11 cells transformed with the pESC vector were cultured in SGI media without tryptophan for 48 h before inoculating YPSE media to an initial OD₆₀₀ of 0.1. After 8 h of incubation, the media was adjusted to 2% galactose and incubated a further 48 h before determining the accumulation of 5-epi-aristolochene in the culture by GC-MS. WAT11 cells transformed with the Yep352 vector were grown similarly in SGI media without supplemental uracil for 48 h before inoculating YPD media containing 0.5% glucose to an initial OD₆₀₀ of 0.1. The accumulation of 5-epi-aristolochene was determined after a further incubation of 48 h.

harboring the TEAS gene (colony PCR) were then tested for growth and production of 5-*epi*-aristolochene. Surprisingly, galactose induction of the cultures grown in glucose-containing media did not accumulate detectable levels of the expected sesquiterpene. Because of possible transcriptional suppression of the galactose-inducible promoter by glucose (Hu et al., 1995), a sucrose-based media was substituted and sesquiterpene production evaluated. Relative to the terpene production levels observations by DeJong et al. (2006); Jackson et al. (2003); and Ro et al. (2006), galactose-inducible sesquiterpene production even under these conditions was still marginal (1 μ g/L) (Table I).

The low level of sesquiterpene accumulation under these conditions could reflect any number of limiting conditions including poor induction of transgene transcription, low specific activity of the introduced synthase activity, limited coupling between the introduced sesquiterpene synthase and the endogenous FPP pools, or even atmospheric losses of the naturally volatile 5-*epi*-aristolochene. Inclusion of solid phase absorbance materials such as Diaion HP-20 to the cultures did not improve yields (Jackson et al., 2003), but use of a modified ADH1 promoter in combination with a glucose-based media did improve sesquiterpene accumulation 200-fold (Table I).

Improving Hydrocarbon Production

While production of hundreds of μ g/L of sesquiterpenes represents significant accumulation, further developments for a more robust production platform to facilitate high resolution chemical identification (i.e., NMR), analysis using small culture volumes (mL vs. L) and high-throughput screens were sought, and in particular, the ability to direct and/or enhance the flux of carbon and isoprenoid intermediates toward sesquiterpene production. DeJong et al. (2006); Jackson et al. (2003); and Ro et al. (2006) reported somewhat contradictory findings when attempting to enhance the pools of particular isoprenoid intermediates that feed directly into sesquiterpene or diterpenes biosynthesis. Upregulated expression of FPP synthase did not substantially improve sesquiterpene accumulation (Jackson et al., 2003; Ro et al., 2006), but introduction of a heterologous GGPP synthase appears to dramatically improve diterpene accumulation (DeJong et al., 2006). Given findings of the homeostatic (regulatory) functions FPP mediates in cells (Gardner and Hampton, 1999) and the observation that sterol accumulation was only elevated 28% in upregulated FPP synthase mutants (Chambon et al., 1991), alternative means for manipulating the FPP pool were sought.

Several yeast lines originally designed and developed by Millis et al. (US patents 6,531,303 and 6,689,593) specifically to enhance FPP levels were examined (Fig. 1). SW24 contains a knockout mutation in the squalene synthase *ERG9* gene and simultaneously was selected for aerobic uptake of sterols (*sue*). The exact locus or mechanism

responsible for the *sue* mutation is not known, but it does appear similar to other such mutants which perturb heme biosynthesis as well (Regnacq et al., 2002). Several phosphatase activities capable of dephosphorylating FPP to farnesol (FOH) have also been identified (Faulkner et al., 1999). CALI7-1 contains an inactivation of one of these genetic loci, *DPPI*, along with the *erg9 Δ ::HIS3* and sterol uptake mutations. Lastly, CALI5-1 contains the *erg9 Δ* , *dpp1*, and *sue* mutations plus several copies of a modified form of the *HMG2* gene coding for a deregulated HMGR enzyme activity. Yeast containing upregulated forms of HMGR, including forms missing an amino-terminal region important for targeting the enzyme to the endomembrane system and putative regulatory domains, can accumulate up to 10-times more squalene than wild-type lines (Donald et al., 1997). These lines were thus first characterized for their growth characteristics and accumulation of FOH, the dephosphorylation product of FPP (Fig. 2A). All three lines grew well in standard nutrient rich broth supplemented with ergosterol and attained about the same culture density. All three lines also accumulated very significant levels of FOH, while no FOH accumulation was detectable in the original parental strain ATCC 28383 (Song, 2003). However, while CALI5-1 and CALI7-1 accumulated maximums of 18–30 mg/L, SW24 accumulated an excess of 80 mg/L. Equally important to note, FOH accumulation in the CALI lines correlated well with early and rapid phases of the culture growth while two-thirds to three-quarters of the FOH accumulating in SW24 occurred after the cultures had reached stationary growth phase.

To gain an appreciation for the sesquiterpene biosynthetic capacity of these different yeast lines, three different sesquiterpene synthase genes were engineered into an ADH1 promoter-yeast expression vector and the recombinant vectors transformed into each of the yeast lines. TEAS (tobacco *epi*-aristolochene synthase), HPS (*Hyoscyamus muticus* premnaspirodiene synthase), and CVS (citrus valencene synthase) were chosen for this evaluation because TEAS and HPS have been extensively characterized (Mathis et al., 1997) and at least in bacterial expression studies, the apparent specific activity of the CVS enzyme is approximately one-tenth that of TEAS or HPS (Greenhagen, 2003). Engineering of these constructs into the CALI5-1 line proved difficult for unknown reasons (perhaps chemical toxicity or molecular incompatibility) and stable CALI5-1 lines with the TEAS and HPS constructs were not obtained. Nonetheless, all the terpene biosynthesizing lines, regardless of the sesquiterpene synthase gene introduced, generated a single dominant sesquiterpene product with no evidence for any secondary transformations mediated by innate yeast metabolism.

SW24 and CALI7-1 engineered with the TEAS gene grew similar to one another, but not as well as the non-transformed controls (Fig. 2B). However, in contrast to the FOH accumulation pattern observed in the control lines (Fig. 2A), 5-*epi*-aristolochene accumulated rapidly during the rapid growth phase and continued to do so even when

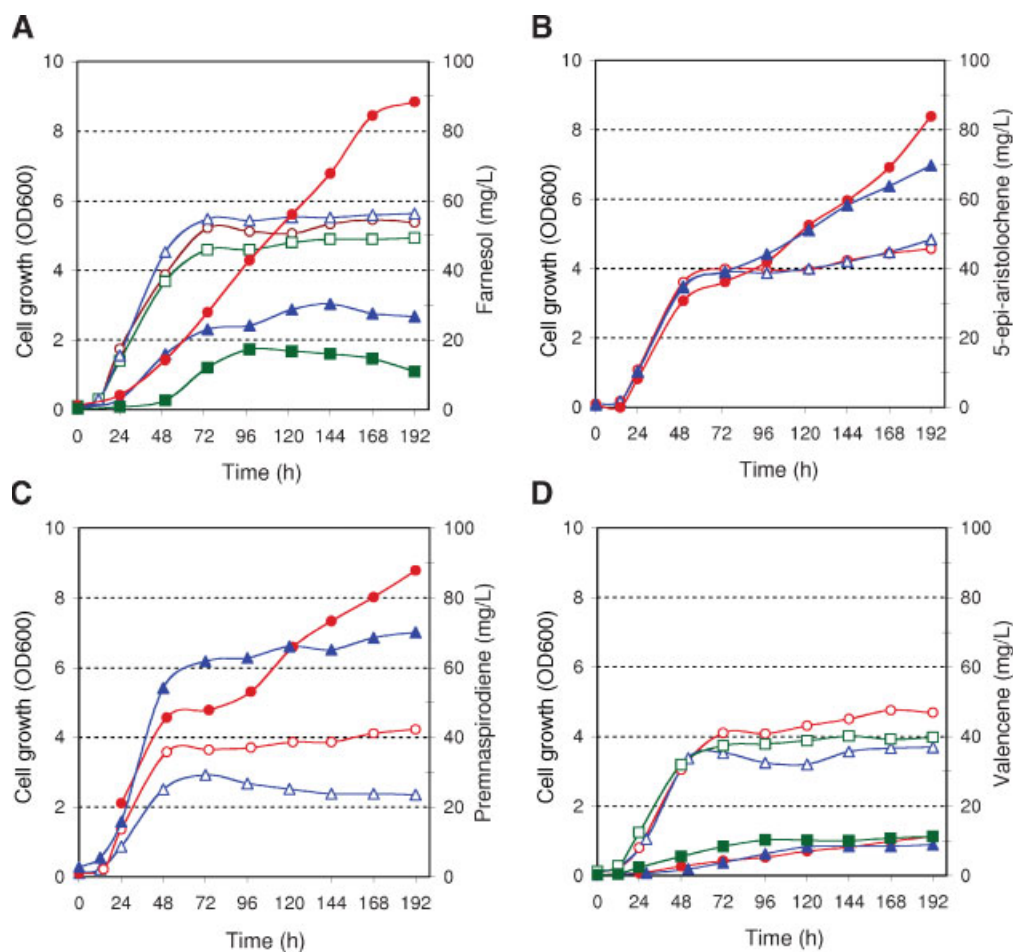


Figure 2. Comparison of cell culture growth and terpene production by yeast strains developed for enhanced levels of FPP and engineered for novel sesquiterpene biosynthesis. Yeast lines developed with specific modifications to the MVA pathway (SW24—*sue, erg9* (circles); CALI7-1—*sue, erg9, dpp1* (triangles); CALI5-1—*sue, erg9, dpp1, HMGR* (squares)), were monitored for cell growth (open symbols) and terpene production (solid symbols). Cell culture growth and farnesol (dephosphorylated form of FPP) accumulation by the initial yeast strains (A) are compared to those strains genetically engineered for 5-epi-aristolochene (B), premnaspirodiene (C), and valencene (D) accumulation. The corresponding terpene synthase genes (TEAS (B); HPS (C); and CVS (D)) were inserted downstream of the ADH1 promoter in the YEp352-URA3 vector and transformed into the respective yeast lines.

the cells entered into stationary phase. Over a 190 h growth cycle, the SW24-TEAS line accumulated almost 90 mg/L of sesquiterpene while the CALI7-1-TEAS line accumulated an excess of 70 mg/L. A similar pattern of terpene accumulation was observed in lines engineered with the HPS gene (Fig. 2C). However, neither the SW24-HPS or CALI7-1-HPS lines attained a cell density as high as the TEAS-harboring lines, yet sesquiterpene accumulation in the CALI7-1-HPS culture reached 70 and 90 mg/L in SW24-HPS culture. All the lines transformed with the CVS gene grew to the same stationary level, approximately 10–20% lower than the control lines, but accumulated only a maximum of 10 mg of valencene per liter within 90–120 h, about one-eighth to one-tenth the level of sesquiterpenes accumulating in the TEAS and HPS lines.

Also important to note is that level of FOH (80 mg/L) accumulating in the control SW24 line (Fig. 2A) is directly comparable to the level of 5-epi-aristolochene or premnaspirodiene accumulating in the SW24 lines engineered with the TEAS or HPS genes, approximately 90 mg/L in both cases. In contrast, the level of FOH accumulating in the control CALI7-1 line (about 30 mg/L) is only about half the level of sesquiterpenes accumulating in the CALI7-1-TEAS and CALI7-1-HPS lines (70 mg/L in each case), suggesting a greater flexibility in the rate of carbon flux through the mevalonate pathway in the CALI7-1 cell lines relative to the SW24 line. Consistent with this notion, independent measurements after 120 h of incubation indicated a 10-fold drop in the FOH levels in the SW24-HPS line to 6 mg/L (relative to 70 mg/L in the SW24 line), but only a 2-fold drop

in the CALI7-1-HPS line to 15 mg/L (relative to 30 mg/L in the CALI7-1 line).

SW24, CALI-5, and CALI-7 are auxotrophic mutants for tryptophan, leucine, and uracil and the results reported in Figure 2 were obtained using the a modified form of the YEp352 vector that restores prototrophic growth in the absence of exogenous uracil. Hence, the engineered lines in Figure 2B were grown in defined media (SCE) supplemented with tryptophan and leucine, while the non-transformed control line was grown in media supplied with all three nutrients (Fig. 2A). However, as noted above, the control lines grew to approximately 20% higher cell densities than the engineered lines suggesting that restoration of prototrophic growth for uracil might not be as efficient as nutrient supplementation and that higher terpene yields might be attainable using an alternative selection marker. The TEAS, HPS, and CVS genes were therefore engineered into a modified pESC vector, which like the YEP vector directs synthase gene expression via an ADH1 promoter, but provides for restoration of leucine, rather than uracil, biosynthesis in the transformed yeast. SW24 lines transformed with all three different terpene synthase genes grew similarly to one another with typical growth kinetics, reaching culture densities corresponding to an OD₆₀₀ of 9 units (Fig. 3). For comparison, SW24 not harboring a transformation vector and grown in media supplemented with both leucine and uracil grew to a density level corresponding to 5 absorbance units (Fig. 2A), while those lines transformed with the YEp352-URA3 vector reached maximum densities of 4–4.5 absorbance units.

Interestingly, sesquiterpene accumulation was moderately improved in the pESC engineered lines over the 216 h of analysis relative to the YEp352 cultures (compare Figs. 2 and 3). Levels of both 5-epi-aristolochene and premnaspirodiene

reached 90 mg/L while valencene production doubled to 20 mg/L. These levels, however, reflect accumulation within a timeframe when the cultures are entering into the early stages of stationary growth (120–144 h of growth), whereas cultures transformed with the YEp vectors reached stationary growth much earlier (48–72 h post-inoculation). Moreover, as evident for the SW24-TEAS and SW24-HPS lines harboring the YEp constructs, upwards of 40–50% more terpene accumulation can occur during a prolonged stationary growth phase of the cultures. However, most significant is the doubling of biomass accumulation in lines transformed with the pESC vector versus the YEp352 vector. pESC restores leucine prototrophic growth, suggestive that leucine supplementation to lines transformed with the YEp352 vector is less than optimal for maximal growth. The improved biomass accumulation nonetheless does not appear to directly translate into additional sesquiterpene hydrocarbon production for the yeast engineered with the TEAS and HPS genes, but did double valencene accumulation in the CVS line (>19 mg/L in Fig. 3 vs. ~10 mg/L in Fig. 2D).

Terpene Hydroxylation

While metabolic engineering to increase the availability of FPP and diversion of this pool of intermediate to sesquiterpene hydrocarbon production is a critical first step in establishing any terpene production platform, introduction of downstream biosynthetic modifications to the hydrocarbon scaffolds is of equal importance because of the structural complexity and biological activity afforded by these modifications (Takahashi et al., 2005). Many of these secondary modifications arise from the regio- and stereochemical insertion of hydroxyl groups into the hydrocarbon scaffolds, which then serve as sites for tertiary modifications like methylation, acetylation, aryl-group addition, and the formation of epoxides and lactones. Hence, efficient coupling of terpene hydroxylation reactions with hydrocarbon production is an essential first step for a more complete recapitulation of the entire terpene biosynthetic repertoire.

Biosynthetic hydroxylation of 5-epi-aristolochene was examined by the co-expression of EAH and a complementary tobacco cytochrome P450 reductase along with TEAS in yeast strain SW24. EAH catalyzes the stereo-specific, stepwise hydroxylation of 5-epi-aristolochene with the first hydroxyl introduced on the β -face at C1, followed by α -hydroxylation at C3 (Takahashi et al., 2005). Careful enzymological characterization of EAH revealed a relative efficient turnover rate for the first hydroxylation at C1, but an even more efficient turnover rate for the second hydroxylation at C3, characterized by a 10-fold lower K_m for the mono-hydroxylated intermediate versus the initial hydrocarbon substrate (Takahashi et al., 2005). One obvious prediction for the efficient in vivo coupling of the hydroxylation reaction with hydrocarbon production would

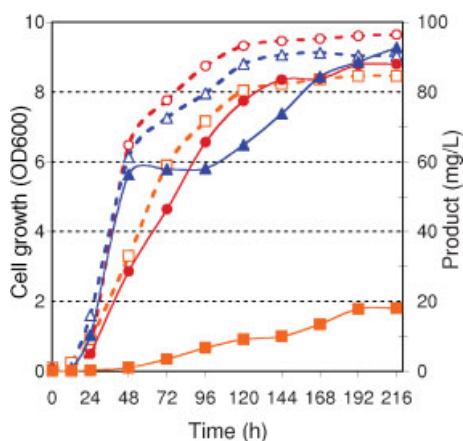
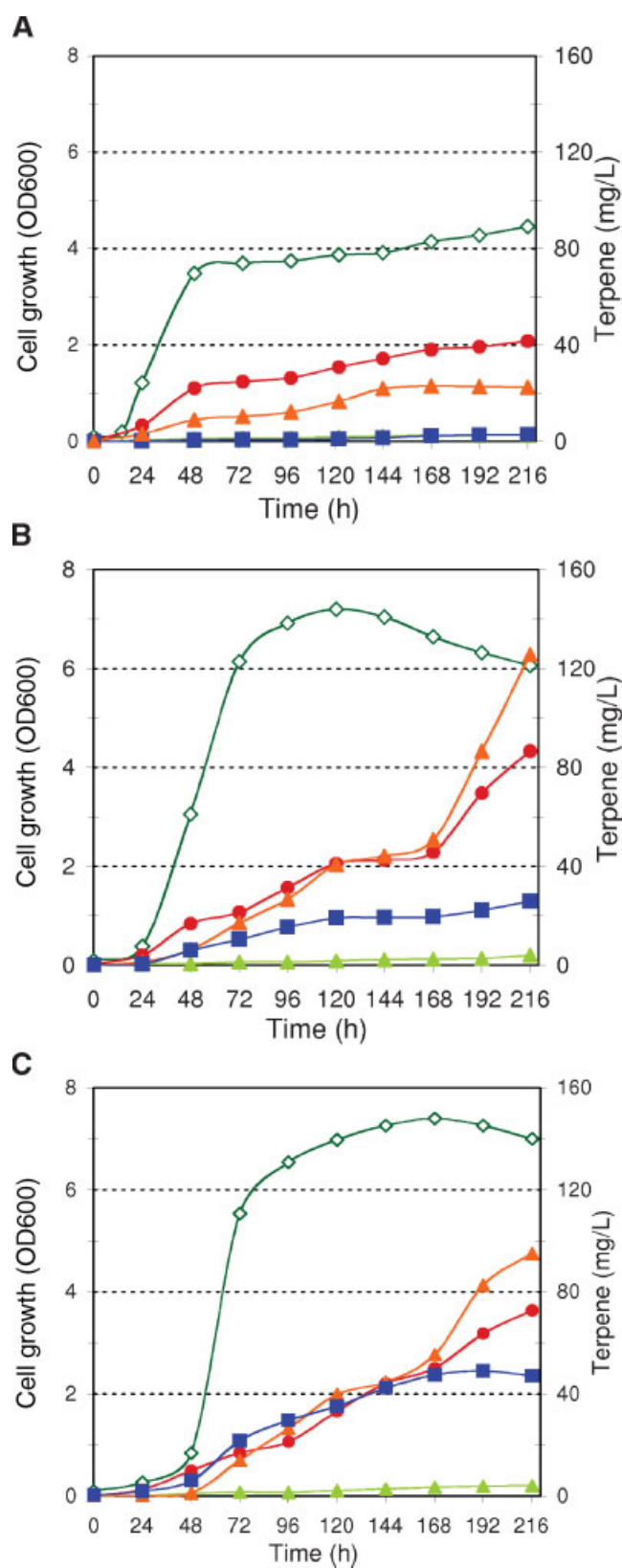


Figure 3. Comparison of cell culture growth (open symbols) and terpene accumulation (solid symbols) by yeast strain SW24 engineered with different terpene synthase genes inserted downstream of the ADH1 promoter in the pESC-LEU2 expression vector. SW24 lines transformed with the TEAS, HPS, or CVS synthase genes were monitored by GC-MS for 5-epi-aristolochene (circles), premnaspirodiene (triangles), or valencene (squares) accumulation, respectively.



herefore be the preferential accumulation of capsidiol, a di-hydroxylated product, rather than the mono-hydroxylated sesquiterpene. Another important consideration for terpene hydroxylation was the absolute requirement of reducing equivalents supplied from NADPH via a cytochrome P450 reductase to the EAH enzyme. In preliminary experiments, no terpene hydroxylase activity was observed in yeast without the simultaneous expression of the tobacco cytochrome P450 reductase gene. Also, because the optimal stoichiometric relationship between TEAS, EAH, and P450 reductase enzymes for efficient di-hydroxylated product formation and difficulties associated with actual means for controlling the levels of the respective enzymes in transgenic yeast, multiple expression vectors with several arrangements of the genes within the vectors were evaluated.

Tandem array of the EAH, EAS, and P450 reductase genes within one expression vector was the least effective design tested for production of hydroxylated sesquiterpenes in the SW24 yeast line, and especially poor when using a URA3 selection marker (Fig. 4A). As noted in earlier experiments, cell culture growth was compromised with this particular selection marker and sesquiterpene hydrocarbon accumulation was approximately 40 mg/L, about 50% of that observed when only the EAS gene was introduced into this yeast line with this particular vector (compare Fig. 2B with Fig. 4A). Very little hydroxylated terpene accumulated in these cultures. Dividing the three genes between the Yep-URA3 and pESC-LEU2 expression vectors restored more vigorous cell culture growth and hydrocarbon accumulation to levels similar to those observed when only the EAS gene was engineered into the SW24 line (compare Figs. 4B and C to 3). However, the dihydroxylated sesquiterpene capsidiol accumulated in both these culture lines in addition to 5-epi-aristolochene. Interestingly, levels of capsidiol accumulation, exceeding 50 mg/L, were directly comparable to hydrocarbon accumulation when the hydroxylase and reductase genes were paired together on a single expression vector (Fig. 4C), versus a 4 to 1 ratio of hydrocarbon to hydroxylated product (approximately 20 mg/L) when EAH and reductase genes were borne on separate expression vectors (Fig. 4B). Also important to note, the accumulation rate of hydrocarbon and oxygenated forms increased in the later stages of the stationary phase (+168 h) of these cultures, which might

Figure 4. Comparison of different gene organization and expression vectors on cell culture growth and terpene accumulation by yeast strain SW24 engineered for hydroxylated sesquiterpene accumulation. SW24 was engineered with 5-epi-aristolochene synthase (TEAS), 5-epi-aristolochene dihydroxylase (EAH), and a tobacco cytochrome P450 reductase in a single YE_p-URA3 expression vector (A), or with TEAS and the reductase genes cloned into the YE_p-URA3 expression vector and EAH into the pESC-LEU2 expression vector (B), or with the reductase and EAH genes cloned into the YE_p-URA3 expression vector and TEAS into the pESC-LEU2 expression vector (C). The respective lines were monitored for cell growth (green diamonds), farnesol (orange triangles), sesquiterpene hydrocarbon (5-epi-aristolochene, red circles), mono-hydroxylated sesquiterpene (1 β -hydroxy-epi-aristolochene, lime triangles), and di-hydroxylated sesquiterpene (capsidiol, blue squares) over a 9-day cell culture cycle.

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reflect a lesser demand for primary metabolism and a greater availability of carbon for the engineered terpene metabolism.

Discussion

There is intense interest in engineering terpene production platforms in fermentable organisms to overcome limited availability of these biologically active and commercially valuable compounds (Ro et al., 2006) and as a means to facilitate high-throughput screening efforts for the molecular evolution of novel and unique terpenes (Carter et al., 2003; DeJong et al., 2006; Jackson et al., 2003; Martin et al., 2003). And several of these investigations have reported significant advances in engineering terpene biosynthesis into bacterial and yeast systems. Carter et al. (2003) and Martin et al. (2003) demonstrated that *E. coli* could be engineered for enhanced terpene hydrocarbon production by either manipulating the innate biosynthetic capacities or by the introduction of an alternative biosynthetic pathway, respectively. Furthermore, Carter et al. (2003) attempted to couple GPP synthase and a monoterpene synthase with downstream modifying enzymes in the menthol biosynthetic pathway in *E. coli*, but only observed generation of hydrocarbon production. They were able to demonstrate that the subsequent enzymes were expressed in the bacterial host and could hydroxylate terpene hydrocarbons fed exogenously to the engineered *E. coli*, but at very low rates, an indication of the limited capacity of a prokaryotic system for a complex eukaryotic pathway.

DeJong et al. (2006); Jackson et al. (2003); and Ro et al. (2006) have provided evidence that yeast too can be advanced for terpene production by manipulating the native mevalonate pathway for either sesquiterpene or diterpene biosynthesis. Jackson et al. (2003) reported 370 μg of epi-cedrol per liter in yeast overexpressing a truncated HMGR activity and epi-cedrol synthase (ECS) in the *upc2-1* genetic background, but little additive effect of overexpressing FPP synthase. In comparison, DeJong et al. (2006) reported 0.7–1 mg of the diterpene taxadiene per liter in yeast overexpressing a GGPP synthase in combination with the corresponding diterpene synthase. Like Carter et al. (2003); DeJong et al. (2006) also included *Taxus* specific P450 hydroxylases and a cytochrome P450 reductase in their engineered yeast lines, observed suitable expression of the corresponding hydroxylase and reductase genes, but did not detect any oxygenated products. Similar to the approach of Jackson et al. (2003); Ro et al. (2006) observed greater than 50 mg/L sesquiterpene hydrocarbon accumulation in yeast engineered for overexpression of a truncated HMGR2 gene in the *upc2-1* genetic background along with the co-expression of amorphadiene synthase. A two to threefold enhancement in hydrocarbon accumulation was observed when a knockout mutation in squalene synthase (ERG9) was included, but little added effect of overexpressing a FPP synthase. Importantly however, these investigators reported the efficient conversion of the sesquiterpene hydrocarbon to

oxygenated product by the co-expression of a corresponding terpene hydroxylase and cytochrome P450 reductase.

The results reported here corroborate and advance yeast as a production platform for terpenes as well. In particular, we investigated three particular mechanisms to increase the endogenous pool of FPP and the means for diverting this intermediate to sesquiterpene biosynthesis (Fig. 1). Limiting the use of the FPP pool for sterol biosynthesis by introducing a knockout mutation of squalene synthase (*erg9*) and simultaneously obtaining a mutant capable of efficient, aerobic uptake of ergosterol (*sue*) from the culture media provided for a very significant enhancement in the FPP pool (as measure by FOH accumulation). Limiting the endogenous dephosphorylation of FPP by knocking out a phosphatase activity (coded by DPP1) known to contribute to the hydrolysis of FPP in yeast (Faulkner et al., 1999), and upregulating the catalytic activity of HMGR, a putative rate-limiting step irreversibly committing carbon to the isoprenoid pathway in general, did not improve sesquiterpene production beyond that of the *erg9/sue* mutations under these growth conditions. Nonetheless, upwards of 100 mg of sesquiterpene were produced per liter, which can be correlated in part with the relative catalytic efficiency of the respective terpene synthase introduced into the yeast line. This notion appears consistent with the observations of Jackson et al. (2003) and Ro et al. (2006). Much less epi-cedrol accumulated in yeast overexpressing ECS (Jackson et al., 2003) relative to artemisinin in similarly engineered yeast overexpressing the amorphadiene synthase (ADS) gene (Ro et al., 2006). Previous biochemical characterizations suggests that the catalytic efficiency (k_{cat}/K_m) of ADS (2.2×10^{-3} , Picaud et al., 2005) is much greater than for ECS (Mercke et al., 1999). Likewise in the current study, the catalytic efficiency of the TEAS and HPS enzymes (0.11–0.54, Mathis et al., 1997) is some 10-fold superior to CVS (Greenhagen, 2003; Niehaus and Chappell, unpublished observations), which may contribute to the lower yields of valencene versus 5-epi-aristolocene or premnaspirodien in yeast over-expressing the respective sesquiterpene synthases. Hence, another means for improving the terpene production capacity of yeast may entail protein engineering for improved catalytic efficiency of the terpene synthases.

Comparison of the levels of FOH and sesquiterpenes accumulated by the various yeast lines (Fig. 2) suggest a greater plasticity in the mevalonate pathway of the CALI7-1 line relative to that in the SW24 cell line. FOH accumulated to 90 mg/L in the control SW24 cell line, while sesquiterpene levels accumulated to similar levels in the engineered SW24-EAS and SW24-HPS lines. FOH levels were also significantly lower in the SW24 lines transformed with the terpene synthase genes, to approximately 6 mg/L. In contrast, FOH levels in the control CALI7-1 line only reached upwards of 30 mg/L, but the level of sesquiterpenes approached 70 mg/L with FOH levels of 15+ mg/L in the CALI7-1-TEAS and CALI7-1-HPS cell cultures. One interpretation of these observations is that the FOH pool size in the CALI7-1 line is

more regulatory because of the *dpp1* mutation. The absence of the encoded DPP1 phosphatase activity leads to a greater percentage of the FOH being in its diphosphorylated form within the cells, and this in turn feedbacks and suppresses the flux of carbon down the general mevalonate pathway (Gardner and Hampton, 1999). Introduction of the highly active terpene synthases, TEAS or HPS, into the CAL17-1 line efficiently diverts the accumulated FPP under these conditions, relieving the feedback inhibition and thus increasing the overall flux rate of carbon through this pathway. A similar phenomenon may not occur in the SW24 background because the cells are conditioned to a relatively low and constant level of FPP. That is most of the FPP is readily dephosphorylated to FOH, in part by the DPP phosphatase, and there is little modulation in the low levels of FPP, even when a terpene synthase activity is introduced. The cells thus maintain a constant flux rate of carbon down the mevalonate pathway.

Our work also illustrates how important the design of the expression vectors are for terpene accumulation. Both Ro et al. (2006) and this study reported the efficient conversion of the terpene hydrocarbon pool to oxygenated products. As shown in Figure 4, constructs physically separating the hydroxylase gene from the reductase gene, either arrayed down a multi-gene construct or on separate expression vectors, yielded very little oxygenated product. However, expression constructs having the reductase gene preceding the hydroxylase gene on the same plasmid provided for an approximate 50% coupling of oxygenation to hydrocarbon production. Such a design must in some way support an optimal stoichiometric expression of the two genes and/or their assembly into active metabolic units. Further improvements in hydrocarbon and oxygenated product yield may obviously result from other combinations of expression vectors, including the use of individual vectors for each gene and integration of multiple gene copies directly in the yeast genome.

Our initial attempts to improve the stoichiometry and coupling of the terpene synthase and hydroxylase enzymes by physically linking the two proteins were unsuccessful (data not shown). Neither amino- or carboxy-terminal fusions of synthase to the terpene hydroxylase expressed well nor exhibited significant hydroxylating activity. Hence, alternative strategies to improve assembly and coupling between the respective synthase, hydroxylase, reductase and additional downstream modifying enzymes may be necessary. One approach would be to identify those domains mediating the physical interaction between the respective proteins, the protein-protein interaction domains, and to further engineer these sites for improved binding and coupling. Alternatively and perhaps more speculative, other factors could mediate the assembly of these enzymatic complexes as recently reported for sterol biosynthesis in yeast (Mo et al., 2002). In comparison to the yeast production platform described here, very little or no sesquiterpene hydrocarbon is evident in plants elicited to coordinately express the TEAS and EAH activities (Watson et al., 1985). Plant cells appear to

produce exclusively oxygenated terpene products. Perhaps the coupling of these enzymatic activities in the native host species arises because of yet other proteins that bring the respective enzymes together in some sort of metabolic channel or unit, proteins that might function in a chaperone type mechanism or could provide a structural scaffold for the assembly of a metabolon. Regardless of the mechanism, the results described here now provide a convenient and facile platform for investigating these possibilities, as well as a platform for the production of industrial and commercial important terpenes.

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