ENGINEERING NOVEL TERPENE PRODUCTION PLATFORMS IN THE YEAST SACCHAROMYCES CEREVISIAE

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ENGINEERING NOVEL TERPENE PRODUCTION PLATFORMS
IN THE YEAST SACCHAROMYCES CEREVISIAE

DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Agriculture at the University of Kentucky

By
Xun Zhuang
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Director: Dr. Joe Chappell, Professor of Plant Physiology
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ABSTRACT OF DISSERTATION

ENGINEERING NOVEL TERPENE PRODUCTION PLATFORMS IN THE YEAST SACCHAROMYCES CEREVISIAE

The chemical diversity and biological activities of terpene and terpenoids have served in the development of new flavors, fragrances, medicines and pesticides. While terpenes are made predominantly by plants and microbes in small amounts and as components of complex mixtures, chemical synthesis of terpenes remains technically challenging, costly and inefficient. In this dissertation, methods to create new yeast lines possessing a dispensable mevalonate biosynthetic pathway wherein carbon flux can be diverted to build any chemical class of terpene product are described. The ability of this line to generate diterpenes was next investigated. Using a 5.5 L fed batch fermentation system, about 569 mg/L kaurene and approximately 207 mg/L abietadiene plus 136 mg/L additional isomers were achieved. To engineer more highly modified diterpenes that might have greater industrial, agricultural or medicinal applications, yeast engineered for kaurenoic acid production reached 514 mg/L with byproducts kaurene and kaurenal accumulating to 71.7mg/L and 20.1mg/L, respectively, in fed batch fermentation conditions. Further genetic modifications yielded additional yeast lines engineered for monoterpene, ZXM, and triterpene, ZXB, production. ZXM, for instance, engineered with a mutant prenyltransferase designed for GPP biosynthesis and a bi-functional monoterpene/sesquiterpene synthase gene from strawberry (FaNES1) accumulated 84.76 mg/L linalool (monoterpene) and 20.54 mg/L nerolidol (sesquiterpene). In contrast, ZXB engineered for triterpene biosynthesis accumulated 297.7 mg/L squalene under shake flask conditions.

KEYWORDS: Saccharomyces cerevisiae, terpene metabolic engineering, diterpene, monoterpene, triterpene
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ENGINEERING NOVEL TERPENE PRODUCTION PLATFORMS
IN THE YEAST SACCHAROMYCES CEREVISIAE

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This thesis would not have come about without the support from my mentor, Joe Chappell. He provided valuable training, advice and long-term encouragement throughout my graduate career. Without his encouragement, I would not have been able to accomplish all the work reported in this thesis. I will forever be grateful to him for helping me become who I am today. I also thank my committee members George Wagner, Ling Yuan, Todd Porter and my outside examiner Barbara Knutson for their support and critical comments. Next, I wish to thank our former lab member Dr. Shuiqin Wu, who gave important suggestions on experimental design and performed the initial work of diterpene engineering in yeast. My achievements would also not have been possible without the help and encouragement of all the Chappell laboratory members. Last but not least, I also thank my family for their continual support of my career.
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Chapter 1: Background and Introduction

1.1. Natural products

Plants, microorganisms and animals produce a large variety of organic chemical compounds, some of which are used universally for growth and metabolism and others seem to play specialized roles in the life cycle of the organism (Maimone & Baran, 2007). As such, two large classes of natural products are widely recognized. Primary metabolites are those essential for life in all eukaryotic organisms, while specialized metabolites appear to give species specific advantages for occupying distinct environmental niches. The distinctive role specialized metabolites play in an organism’s natural history, for example how these metabolites provide protection against microbial challenge, have also not escaped attention for their possible utility in a wide range of applications. For example, many of the currently used drugs are derived from or inspired by plant-derived specialized chemicals and are commonly referred to as Natural Products (Newman & Cragg, 2012). Capturing the all the possible chemical and structural diversity of Natural Products has recently been identified as a major objective within the scientific community in large part because of the wide array of applications Natural Products have and the resulting economic implications.

1.2. Terpenoids

Terpenes and terpenoids are a large and diverse family of Natural Products with more than 55,000 having been identified (Maimone & Baran, 2007). However, based on the biosynthetic mechanisms responsible for terpenes, chemists have predicted that only a small fraction of all the possible terpene compounds have been discovered (Bouvier et al., 2005). Terpenes are derived from a five-carbon isoprene building block with different combinations of the isoprene units generating different classes of the terpene products. The classification and biosynthesis of terpenoids are based on the number of five-carbon
units they contain as illustrated in Fig.1.1. Monoterpenes (consisting of 10 carbons), sesquiterpenes (15 carbon derivatives), and diterpenes (20 carbon derivatives), arise from the corresponding intermediates geranyl diphosphate (GPP), farnesyl diphosphate (FPP), and geranylgeranyl diphosphate (GGPP). These intermediates in turn arise by the sequential head to tail condensation of C5 units. Higher order terpenes like triterpene (30 carbons) are formed from two farnesyl units condensed head-to-head (Buchanan et al., 2002).

Monoterpenes are well known as the volatile essence of flowers and such mixtures can account for up to 5% of plant dry weight (Buchanan et al., 2002). Menthol and camphor are common monoterpenes found in diverse plant families and whose structural complexity in terms of stereo- and regio-chemistry are emphasized in Fig. 1.2. Besides providing pleasing fragrances, monoterpenes have been shown to function as signal molecules in defense mechanisms against pathogens (Hick et al., 1999). Monoterpenes have commercial values as flavors, fragrances, essential oils, and as anticancer and antimicrobial drugs (Burke et al., 1997). Sesquiterpenes (C15) are also found in essential oils, and many sesquiterpenes possess antibiotic activities, prompting suggestions that they are produced by plants as phytoalexins through a defense mechanism (Bailey, 1982). Diterpenes (C20) include gibberellins (plant hormones), vitamin A, as well as pharmaceutical important metabolites such as taxol, an exceptional anticancer regent (Barkovich & Liao, 2001). Triterpenes (C30) include the brassinosteroids, phytosterols important for lipid membrane composition, and components of surface waxes, such as oleanolic acid of grapes. Squalene, the major content of shark liver oil, is a linear triterpene and common ingredient in cosmetic products (Huang et al., 2009), has special utility as a lubricant for high performance machinery, and is a common adjuvant in many pharmaceutical formulations (Bhilwade et al., 2010, Huang et al., 2009, Reddy & Couvreur, 2009). Tetraterpenes (C40) include carotenoid accessory pigments, like lycopene, which assist and support the light
reactions of photosynthesis. Longer chain terpenes, so-called polyterpenes, contain more than 8 isoprene units and include examples like ubiquinone and rubber (Buchanan et al., 2002).

1.3. Terpenoid metabolic pathways

There are two pathways for terpene biosynthesis in plant cells. One is the mevalonate pathway pathway (MVA) which is well established and discovered in the 1960s (Bouvier et al., 2005). The other is the mevalonate independent pathway, or more properly referred to as the mevalonate-independent pathway (MEP), which was more recently discovered (Bouvier et al., 2005). The MEP pathway was first discovered in prokaryote cells, and then confirmed to exist in plant cells (Barkovich & Liao, 2001). Interestingly, plants utilize these two pathways to meet different terpene biosynthetic needs.

Sesquiterpenes, sterols, triterpenes and oligoterpenes (side chain of dolichols) are synthesized in the cytosol via the MVA pathway, while monoterpenes, diterpenes, teraterpenes, and polyterpenoids are synthesized in chloroplasts via the MEP pathway using pyruvate and glyceraldehydes-3-phosphate as the primary precursors (Fig. 1.2).
<table>
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<td><strong>Monoterpenoids</strong></td>
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<td>Flavors, fragrances, cleaning products, anticancer, antibacterial, antioxidant, essential oil, biofuel</td>
<td>Limonene, menthol, camphor, linalool</td>
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<td><strong>Sesquiterpenoids</strong></td>
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<td>Flavors, fragrances, pharmaceuticals (antibacterial, antifungal), insecticides, biofuels</td>
<td>Nootkatone, artemisinin, patchoulol, nerolidol, farnesol, capsidol, farnesene, bisabolene</td>
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<tr>
<td><strong>Diterpenoids</strong></td>
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<td>Gibberellins, phytol, taxol, kaurene, abietadiene, kaurenoic acid, abietic acid</td>
</tr>
<tr>
<td><strong>Triterpenoids</strong></td>
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<td>Biologic markers, biofuel, skin moisturizers in cosmetics, immunologic adjuvant in vaccines</td>
<td>Sterols, hopanoids, squalene, botryococcene.</td>
</tr>
<tr>
<td><strong>Tetraterpenoids</strong></td>
<td>Antioxidants, photosynthetic components, pigments, and nutritional elements (vitamins)</td>
<td>Food additives, colorants, antioxidants</td>
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</table>
Figure 1.1. Classification and biosynthesis of isoprenoids. Isoprenoids are biosynthesized from the basic five carbon units IPP and DMAPP.
Figure 1.2. Schematic outline of the two terpene biosynthetic pathways that operate in plants (the MVA and MEP pathways) (Chappell, 2002), their intracellular locations, and examples of the chemical compounds derived from each.
1.4. The mevalonate pathway in *Saccharomyces cerevisiae*

The principal product of the mevalonate pathway is sterols, for example cholesterol in animal cells, stigmasterol and campesterol in plant cells, and ergosterol in fungi, which all play essential roles in establishing the structural integrity of membranes, establishing permeability and fluidity, and also serving as signal compounds in cellular communication (Buchanan et al., 2002). In *Saccharomyces cerevisiae*, only the mevalonate pathway is known to operate and no components of the MEP pathway have been found (Maury et al., 2005). Figure 1.3 shows the intermediates and the related genes involved in the yeast mevalonate pathway (Maury et al., 2005). Two molecules of acetyl-CoA are condensed by acetoacetyl-CoA thiolase, which is encoded by *ERG10*, to synthesize acetoacetyl-CoA. A second condensation reaction between acetoacetyl-CoA and acetyl-CoA is then catalyzed by HMG-CoA synthase encoded by *ERG13* to yield 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA).

HMG-CoA is reduced by HMG-CoA reductase to yield mevalonate. This reaction is catalyzed by HMG-CoA reductase, which is encoded by 2 separate loci in yeast. Both loci appear to compensate for a knockout loss of the other gene. The C5 position of mevalonate is phosphorylated by mevalonate kinase, encoded by *ERG12*. Then a second kinase, phosphomevalonate kinase, encoded by *ERG8*, catalyzes the successive phosphorylation to yield diphosphomevalonate. In the next step the diphosphomevalonate is converted into IPP (isopentenyl diphosphate) by mevalonate diphosphate decarboxylase, encoded by *ERG19*. IPP isomerase, encoded by *IDI1* converts IPP into DMAPP (dimethylallyl diphosphate). The condensation of the C5 building blocks of IPP and DMAPP into FPP is catalyzed by FPP synthase, which is encoded by *ERG20*. FPP can then be used as substrate for sterol and other isoprenoid biosynthetic needs.
Recent studies have discovered that FPP is also available in yeast mitochondria, as evidenced by increasing novel sesquiterpene production three-times by targeting a sesquiterpene synthase to the mitochondria compartment compared with targeting this same enzyme to the cytosol (Farhi et al., 2011). The origin of FPP in mitochondria could be the IPP and DMAPP arising in cytosol being imported and converted in the mitochondria to FPP. Alternatively, a hypothetical leucine metabolism model for the formation of terpene in S. cerevisiae is also a possibility. The leucine catabolism pathway (MCC pathway) is known to occur in the mitochondria of other eukaryotic mammalian and plant cells (Anderson et al., 1998), in mitochondria leucine metabolite to form 3-Hydroxy-3-methylglutaryl-CoA, which can be catalyzed by HMGR to produce mevalonic acid, and then produce IPP and DMAPP through MVA pathway as shown in Fig 1.4 (Carrau et al., 2005). Interestingly, a yeast line engineered with a chimeric diterpene synthase targeted to the cytoplasm along with prenyltransferases streamlined for GGPP biosynthesis, yielded 2-3 times more diterpene when the expression vector also provided a leu2 auxotrophic selection marker gene. The interpretation provided by the authors was that the extra leucine produced by the auxotrophic selection marker gene provided another source for IPP via the leucine catabolic pathway (Fig. 1.4). (Zhou et al., 2012).

1.5. The prenyltransferase and terpene synthase reaction

Prenyltransferases generate allylic diphosphate esters GPP, FPP, and GGPP. These compounds can undergo a variety of reactions, which include cyclization reactions catalyzed by terpene synthases, yielding diverse terpenes based on regio- and stereo-chemical constraints built into the reactions. Prenyltransferases and terpene synthases utilize electrophilic reaction mechanisms to mediate the catalytic reactions (Ohnuma et al., 1996) and typically share a conserved aspartate-rich DDXXD motif thought important for the initial substrate binding and metal-dependent ionization step leading to the first reaction carbocation intermediates. In the prenyltransferase reactions, the allylic
diphosphate ester can be ionized to form a carbocation, then condensed with a second IPP in another round of elongation.

One of the simpler monoterpen synthase reactions is catalyzed by limonene synthase, an enzyme and reaction that has served as a model for all other terpene cyclization reactions as illustrated in Fig. 1.5. The ionization of GPP, assisted by divalent metal ions, provides for a delocalized carbocation and relocation of the diphosphate anion pair to C3, which leads to the allelic isomer LPP (linalyl diphosphate). Rotation about the C2-C3 single bond and a second ionization of LPP promotes the formation of another carbocation intermediate that can cyclize to form the final product limonene.
Figure 1.3. The mevalonate pathway for ergosterol biosynthesis in yeast (*S. cerevisiae*).

The enzymes encoded by the different genes are: *ERG10*: acetoacetyl-CoA thiolase; *ERG13*: HMG-CoA synthase; *HMG1*, *HMG2*: HMG-CoA reductases; *ERG12*: mevalonate kinase; *ERG8*: phosphomevalonate kinase; *ERG19*: diphosphomevalonate decarboxylase; *IDI1*: IPP, DMAPP isomerase; *ERG20*: FPP synthase; *ERG9*: squalene synthase; *ERG1*: squalene epoxidase; *ERG7*: lanosterol synthase.
Figure 1.4. The proposed alternative HMG-CoA formation pathway through leucine catabolism pathway, adapted from (Carrau et al., 2005, Zhou et al., 2012).
Figure 1.5. Limonene synthase model for terpene cyclization.
1.6. Metabolic engineering

Metabolic engineering aims to make genetic modifications in a way to optimize cellular metabolism of microbial or eukaryotic host cells to produce desired novel and specific molecules that are of utility as chemical precursors, pharmaceuticals, fine chemicals, fuels, and food ingredients. Many of the target compounds are normally difficult to produce by organic chemical synthesis and metabolic engineering takes advantage of the specificity of enzyme-mediated biosynthesis to generate stereo-chemically pure products from cheap raw materials.

1.7. Terpene engineering

Terpenes can be obtained by isolation from natural source, metabolic engineering native host, chemical synthesis and fermentation of metabolically engineered cell cultures of bacteria and yeast. Terpenes are usually made by plants and microbes in small amounts and as complex mixtures, making the isolation of single entity molecules difficult. Chemical synthesis of terpenoids is often prohibitively expensive and inefficient because of difficulties in retaining specific configurations around stereo-centers and obtaining absolute purity. Metabolic engineering of terpenes in plant and microbes have hence challenged scientists to aim towards the accumulation of specific, high-value target molecules.

An especially exciting example of microbial engineering for terpenes is the production of the antimalarial drug Artemisinin precursor amorphadiene in engineered yeast with yields in excess of 100 mg/L (Ro et al., 2006) (Table 1.3) and 105 mg/L in E. coli (Martin et al., 2003) (Table 1.2). Further optimization of a two-phase fermentation of amorphadiene producing E. coli strain dramatically increased sesquiterpene production to 500 mg/L (Newman et al., 2006) (Table 1.2). By tethering the enzymes of the MEV pathway (AtoB, HMGS, HMGR) to a synthetic protein scaffold, a further 77-fold enhancement of mevalonate production was observed, indicating the importance of controlling/directing carbon flux through this metabolic pathway in E. coli (Dueber et al., 2009). Additional optimization of fermentation conditions including nitrogen delivery, medium and feeding control strategy led to amorphadiene production >25 g/L in fed-batch bioreactor fermentation (Tsuruta et al., 2009). Amorphadiene or artemisinic acid engineering in yeast have also been advanced significantly in the past few years. By using the leu2d selection marker to maintaining high plasmid copy number in the yeast
strains of Ro et al. (2006), accumulation of amorphadiene and artemisinic acid up to 780 mg/L and 250 mg/L, respectively, in shake flask cultures have been reported (Ro et al., 2008). In other sesquiterpene engineering studies, the Chappell laboratory reported another yeast strain engineered for high level FPP production had the capacity to divert upwards of 50% of this pool to sesquiterpene biosynthesis at yields of 80 mg/L (Takahashi et al., 2007) (Table 1.3).

Taxadiene is a diterpene hydrocarbon and the key intermediate in the biosynthesis of paclitaxel, which is widely used in cancer chemotherapy. Metabolic engineering of taxadiene synthase has resulted in 1.3 mg/L in E. coli (Huang et al., 2001) (Table 1.2) and 1 mg/L in yeast (Dejong et al., 2006) (Table 1.3). Recently, advance improvement in taxadiene engineering achieved 1g/L in E. coli (Ajikumar et al., 2010) under fed-batch fermentation through a multivariate-modular approach for optimize balancing and channeling the flux of IPP and DMAPP toward taxidiene biosynthesis (Table 1.2). More recent approaches in the production level of diterpenes are shown in Table 1.3. By overexpression of fusion enzymes to increase the association of relative enzymes and channeling intermediates to efficient desired products, 238.8 mg/L geranylgeraniol were produced in yeast shake flask cultures (Tokuhiro et al., 2009). Using similar strategies for miltiradiene, the precursor to the Chinese herb medicine tanshionones, up to ~480 mg/L in optimized yeast fermentation was attained (Zhou et al., 2012, Dai et al., 2012).

Monoterpene engineering in E. coli was developed with 5 mg/L (Reiling et al., 2004) and 5 mg/L in yeast (Fischer et al., 2011). Compared with sesquiterpene engineering level in microbial hosts, monoterpene level is quite low, which may reflect limitations of intermediates like GPP.

Concurrent to the microbial genetic engineering, significant progress has also been achieved in the metabolic engineering of terpene metabolism in plants. Work in the Chappell lab has revealed that the redirection of cytosolic or plastidic isoprenoid precursors through over-expression an avian farnesyl diphosphate synthase and an appropriate terpene synthase elevates sesquiterpene production in planta more than 1,000 times higher, with amorphadiene accumulating to 25 µg/g FW and patchoulol to 30 µg/g FW (Wu et al., 2006) (Table 1.4). When a strawberry linalool/nerolidol synthase was over-expressed in Arabidopsis, total terpene levels of 1.5 µg/g FW were observed, and large amounts of linalool were released as volatiles from the transgenic plant, with
the highest level reaching 13.3 µg per plant per day (Aharoni et al., 2003) (Table 1.4). The recent studies in Chappell lab have revealed that targeting FPP synthase and squalene synthase to the chloroplast compartment of secretory trichomes resulted in upwards of 1,760 µg/g FW, or approximately 1% of the dry weight, in greenhouse grown tobacco plants (Wu et al., 2012).

The yeast *Saccharomyces cerevisiae* has several properties make it having tremendous advantages as a host cell to metabolic engineering terpene compounds. As a eukaryote, yeast is a suitable host organism for the high-level production of soluble cytosolic proteins as well as complicated protein or organic molecules with further modification such as glycosylation, which make yeast more advantage than bacteria (Mendoza-Vega et al., 1994). Yeast can also be grown rapidly and reach high cell density on simple media. And genetic manipulations are more readily performed than with other eukaryote hosts. Furthermore, having the native ergosterol biosynthesis pathway with important terpene biosynthesis intermediates and precursors, yeast has more advantage for terpene engineering.

I have illustrated here why terpene compounds have attracted so much attention, and outlined the latest achievements of engineering terpenoids in bacteria, yeast and plant organisms, and as well as the advantages of yeast as host cell factory for engineering highly desired, single entity valuable terpene compounds. The current metabolic engineering efforts for terpene production in yeast have mostly focused on producing specific compounds, especially the sesquiterpene amorphadiene. In our strategies, we have proposed that the yeast baseline FPP pool can be improved by building a yeast line having a dispensable MVA biosynthetic pathway and complete *erg9* knock out instead of using a suppressor promoter to conditional inhibiting *ERG9* gene, as described in Chapter 2. And theoretically, the flux of carbon to farnesyl diphosphate could be utilized and diverted for the biosynthesis of any class of terpene products. With further modifications through molecular and genetic manipulations of this new yeast system, we successfully built all classes of terpene in our newly developed yeast system: for diterpenes as described in Chapter 3, monoterpenes in Chapter 4, and triterpenes in Chapter 5. Hence, a general novel yeast platform suitable for producing all classes of terpene have been built to provide yeast strains suitable for manufacturing of single entity, high-value terpene compounds.
Table 1.2. Summary of Terpene Production in *E. coli* with Different Approaches for Improving the Production Yield.

<table>
<thead>
<tr>
<th>Literature</th>
<th>Isoprenoid produced</th>
<th>Class</th>
<th>Approach</th>
<th>Production yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Carter et al., 2003)</td>
<td>Limonene, carveol, carvone</td>
<td>Monoterpene</td>
<td>Inducible overexpression of heterologous GPP synthase and IPP isomerase genes and feeding of precursor.</td>
<td>5mg/L limonene, No carveol or carvone detect</td>
</tr>
<tr>
<td>(Reiling et al., 2004)</td>
<td>Carene</td>
<td>Monoterpene</td>
<td>Inducible overexpression of heterologous DXS, IDI, and variant of IspA (FPP synthase) that produce GPP.</td>
<td>0.42 µg/OD&lt;sub&gt;600&lt;/sub&gt;/L/h</td>
</tr>
<tr>
<td>(Martin et al., 2003)</td>
<td>Amorpha-4,11-diene</td>
<td>Sesquiterpene</td>
<td>Expression of the genes from <em>S. cerevisiae</em> encoding the mevalonate pathway and amorpha-4, 11-synthase.</td>
<td>112 mg/L</td>
</tr>
<tr>
<td>(Newman et al., 2006)</td>
<td>Amorpha-4,11-diene</td>
<td>Sesquiterpene</td>
<td>Optimization of two-phase partitioning bioreactor for fermentation of amorpha-4, 11 diene producing strain of Martin et al. (2003).</td>
<td>500mg/L</td>
</tr>
<tr>
<td>(Chang et al., 2007)</td>
<td>8-hydroxycadinene and artemisinic acid</td>
<td>Sesquiterpene</td>
<td>Expression of plant cytochrome P450s followed by optimization in strain of Martin et al. (2003).</td>
<td>105 mg/L and 105 mg/L</td>
</tr>
<tr>
<td>(Pfleger et al., 2006)</td>
<td>Mevalonate</td>
<td>Upstream</td>
<td>Developed a method for tuning the expression of multiple genes within operons by generating libraries of tunable intergenic regions (TIGRS) by using of mRNA secondary structures to control production of the first three enzyme in the mevalonate pathway (AtoB, HmgS,HmgR), strain of Martin et al. (2003).</td>
<td>7-fold increase of mevalonate</td>
</tr>
<tr>
<td>(Dueber et al., 2009)</td>
<td>Mevalonate</td>
<td>Upstream</td>
<td>Increase carbon flux through the metabolic pathway by tethering the enzymes in the mevalonate pathway by tethering the enzymes in the mevalonate pathway.</td>
<td>77-fold increase in mevalonate</td>
</tr>
</tbody>
</table>
Table 1.2 (continued)

<table>
<thead>
<tr>
<th>(Tsuruta et al., 2009)</th>
<th>Amorpha-4,11-diene</th>
<th>Sesquiterpene</th>
<th>Optimization of nitrogen delivery and fermentation conditions of strain of Dueber et al. (2009).</th>
<th>&gt;25 g/L in fed-bath bioreactor fermentation</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Huang et al., 2001)</td>
<td>Taxadiene</td>
<td>Diterpene</td>
<td>Overproduction of DXP synthase, IDI, GGPP synthase and taxadiene synthase.</td>
<td>1.3 mg/L</td>
</tr>
<tr>
<td>(Ajikumar et al., 2010)</td>
<td>Taxadiene</td>
<td>Diterpene</td>
<td>Multivariate-modular approach for optimize balancing and channeling the overflow flux from IPP DMAPP toward Taxol biosynthesis.</td>
<td>1g/L Taxadiene and 60mg/L Taxadien-5α-ol fed bath fermentation</td>
</tr>
</tbody>
</table>

Table 1.2 (continued)
### Table 1.3. Summary of Terpene Engineering in yeast (S. cerevisiae) with Different Approaches for Improving the Production Yield.

<table>
<thead>
<tr>
<th>Literature</th>
<th>Isoprenoid produced</th>
<th>Class</th>
<th>Approach</th>
<th>Production yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Carrau et al., 2005)</td>
<td>Linalool, α-terpieneol, citronellol</td>
<td>Monoterpene</td>
<td>Wine yeast under high concentration of assimilable nitrogen fermentation.</td>
<td>&lt;5 µg/L</td>
</tr>
<tr>
<td>(Oswald et al., 2007)</td>
<td>Geraniol, linalool</td>
<td>Monoterpene</td>
<td>Expression of geraniol synthase in yeast mutants defective in ERG20 gene (FPP synthase) K197E.</td>
<td>0.5-1 mg/L</td>
</tr>
<tr>
<td>(Fischer et al., 2011)</td>
<td>Geraniol</td>
<td>Monoterpene</td>
<td>Expression of geraniol synthase in a set of yeast mutants defective in ERG20 gene (FPP synthase) at position K197.</td>
<td>5 mg/L</td>
</tr>
<tr>
<td>(Jackson et al., 2003)</td>
<td>8-epi-cedrol</td>
<td>Sesquiterpene</td>
<td>Over production of epi-cedrol synthase, yeast FPP synthase ERG20 and HMGR.</td>
<td>0.37 mg/L</td>
</tr>
<tr>
<td>(Lindahl et al., 2006)</td>
<td>Amorpha-4,11-diene</td>
<td>Sesquiterpene</td>
<td>Expression of heterologous gene encoding amorpha-4,11-diene synthase on high-copy plasmid and integration in the chromosome.</td>
<td>0.6 mg/L by plasmid and 0.1 mg/L by genome integration transformant</td>
</tr>
<tr>
<td>(Ro et al., 2006)</td>
<td>Amorpha-4,11-diene and artemisinic acid</td>
<td>Sesquiterpene</td>
<td>Expression of heterologous ADS genes combined with overexpression of thHMG gene, down regulating the ERG9 gene by substitution of ERG9 promoter for MET3 promoter, overexpression of upc2-1, integration of additional thHMG, overexpression of ERG20, Co-expression CPR and P450 and amorphaadiene oxidase CYP71AV1 in high</td>
<td>153 mg/L amorphaadiene, and 32 mg/L artemisinic acid in shake flask. 115 mg/L artemisinic acid in bioreactor fermentation.</td>
</tr>
</tbody>
</table>
amorphadiene production yeast line.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Compound(s)</th>
<th>Type</th>
<th>Description</th>
<th>Yield/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Shiba et al., 2007)</td>
<td>Amorpha-4,11-diene, sesquiterpene</td>
<td>Overexpressing native ALD6 and mutated Acs from Salmonella enterica in the strains from Ro et al. (2006) to generate the pyruvate dehydrogenase bypass in yeast.</td>
<td>120 mg/L</td>
<td></td>
</tr>
<tr>
<td>(Ro et al., 2008)</td>
<td>Amorpha-4,11-diene, artemisinic acid</td>
<td>Using Leu2d selection marker vector to heterologous expression ADS and amorphadiene synthase to maintain plasmid stable in yeast and appropriate medium along with optimizing fermentation conditions.</td>
<td>780 mg/L in shake flask. Artemisinic acid 250 mg/L in shake flask and 1 g/L in bioreactor.</td>
<td></td>
</tr>
<tr>
<td>(Asadollahi et al., 2008)</td>
<td>Valencene, cubebol and patchouliol</td>
<td>Downregulating the ERG9 gene by substitution of ERG9 promoter for MET3 promoter and expression of heterologous terpene synthase genes.</td>
<td>3,000 μg/L, 1,600 μg/L and 11,500 μg/L (nonspecific synthases results in a mix of isoprenoids)</td>
<td></td>
</tr>
<tr>
<td>(Takahashi et al., 2007)</td>
<td>5-epi-aristolochene, sesquiterpene</td>
<td>Expression of TEAS, CVS, HPS in erg9 knockout yeast. Co-expression of TEAS, EAH and P450 reductase to hydroxylated of 5-epi-aristolochene.</td>
<td>80 mg/L 5-epi-aristolochene 50 mg/L valencene , 90 mg/L premnaspirodiene and 120 mg/L hydroxylated aristolochene.</td>
<td></td>
</tr>
<tr>
<td>(Dejong et al., 2006)</td>
<td>Taxadiene and taxadien-5α-ol</td>
<td>Heterologous expression of five sequential paclitaxel pathway genes.</td>
<td>taxadiene 1 mg/L and (taxadien-5α-ol) ~25 μg/L</td>
<td></td>
</tr>
<tr>
<td>Source</td>
<td>Compound</td>
<td>Class</td>
<td>Description</td>
<td>Yield (shake flask/bioreactor)</td>
</tr>
<tr>
<td>-------------------------------</td>
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<td>-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
<td>-------------------------------</td>
</tr>
<tr>
<td>(Tokuhiro et al., 2009)</td>
<td>Geranylgeraniol/</td>
<td>Diterpene</td>
<td>Overexpression HMG1 leads squalene accumulation. Overexpression BTS1-DPP1 fusion gene along with HMG1 shift production from squalene to GGOH. Additional BTS1-ERG20 fusion gene increases the GGOH production.</td>
<td>Squalene 191 mg/L, GGOH 228.8 mg/L in shake flask. 3 g/L GGOH in fermentor.</td>
</tr>
<tr>
<td>(Zhou et al., 2012)</td>
<td>Miltiradiene</td>
<td>Diterpene</td>
<td>Overexpression of tHMGR1, BTS1-ERG20 fusion and SmKSL-SmCPS fusion along with complementary leu2 auxotrophic marker and convert yeast from haploid to diploid.</td>
<td>12 mg/L in shake flask, 365 mg/L in bioreactor.</td>
</tr>
<tr>
<td>(Dai et al., 2012)</td>
<td>Miltiradiene</td>
<td>Diterpene</td>
<td>Overexpression of SmCPS and SmKSL along with fusion BTS1-ERG20 in yeast. Switch auxotrophic marker to antibiotic maker on the episomal expression vector increase miltiradiene production.</td>
<td>8.8 mg/L or 62 mg/L by auxotrophic vector or antibiotic vector in shake flask, 488 mg/L in bioreactor.</td>
</tr>
<tr>
<td>(Niehaus et al., 2011)</td>
<td>Botryococcene</td>
<td>Triterpene</td>
<td>Overexpression of fusion gen SSL1-SSL3 (Squalene synthase like gene) in erg9 knock out yeast line.</td>
<td>70 mg/L</td>
</tr>
</tbody>
</table>
Table 1.4. Summary of Terpene Engineering in Plants with Different Approaches for Improving the Production Yield.

<table>
<thead>
<tr>
<th>Literature</th>
<th>Isoprenoid produced</th>
<th>Engineered species</th>
<th>Class</th>
<th>Approach</th>
<th>Production yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Ohara et al., 2003)</td>
<td>Limonene</td>
<td>Tobacco</td>
<td>Monoterpene</td>
<td>Limonene synthase of perilla frutescens were introduced into tobacco to</td>
<td>Plastid localized 143 ng/g FW</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>target in either plastid, cytosol or the endoplasmic reticulum (ER)</td>
<td>390ng/(plant*month) , Cytosol localized 40 ng/g FW</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>515 ng/(plant*month) ER localized 0 ng/g FW</td>
</tr>
<tr>
<td>(Aharoni et al., 2003)</td>
<td>Linalool and</td>
<td>Arabidopsis</td>
<td>Monoterpene</td>
<td>Overexpression of a dual linalool/nerolidol synthase (FaNES1) in plastids.</td>
<td>Total terpenoid 1,500 ng/g FW</td>
</tr>
<tr>
<td></td>
<td>nerolidol</td>
<td></td>
<td></td>
<td></td>
<td>LW Linalool 13,300 ng/day</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Nerolidol 160 ng/day</td>
</tr>
<tr>
<td>(Lücker et al., 2001)</td>
<td>S-linalyl-β-Dglucopyranoside</td>
<td>Petunia</td>
<td>Monoterpene</td>
<td>Petunia hybrid was transformed with a <em>Clarkia breweri</em> S-linalool synthase.</td>
<td>5,000 ng/g FW 14.7 ng/(g day)</td>
</tr>
<tr>
<td>(Lücker et al., 2004)</td>
<td>β-pinene, limonene, and γ-terpinene</td>
<td>Tobacco</td>
<td>Monoterpene</td>
<td>Tobacco plants were engineered with three monoterpene synthase β-pinene,</td>
<td>β-pinene 378 ng/(g·day)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>limonene, and γ-terpinene synthase directed to plastids.</td>
<td>γ-terpinene 630 ng/(g·day)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Limonene 1,206 ng/(g·day)</td>
</tr>
<tr>
<td>(Wu et al., 2006)</td>
<td>Limonene</td>
<td>Tobacco</td>
<td>Monoterpene</td>
<td>Redirection of cytosolic or plastid isoprenoid precursors. overexpression</td>
<td>Limonene: 500 ng/g FW</td>
</tr>
<tr>
<td></td>
<td>Amorpha-4,11-diene</td>
<td></td>
<td></td>
<td>geranyl diphosphate synthase and limonene synthase.</td>
<td>Amorpha-4,11-diene: 25,000 ng/g FW; Patchoulol 30,000 ng/g FW</td>
</tr>
<tr>
<td></td>
<td>Patchoulol</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Study</td>
<td>Product Description</td>
<td></td>
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<td>------------------------------</td>
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<td></td>
</tr>
<tr>
<td>(Zhang et al., 2011)</td>
<td>Introducing amorpha-4, 11-diene synthase in cytosol.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Besumbes et al., 2004)</td>
<td>Heterologous expression of amorpha-4, 11-diene synthase and CYP71AV1. And additional expression of artemisinic aldehyde double bond reductase with or without aldehyde dehydrogenase A</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Wu et al., 2012)</td>
<td>Directing FPS and SQS (squalene synthase) to the secretory trichome cytoplasm or chloroplast.</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
Chapter 2: Building a Terpene Production Platform In Yeast

2.1. Summary

Terpene natural products have attracted keen interest since the 15th century when they served as highly desired trade items by the new world explorers. In more recent times, the chemical diversity and biological activities of this intriguing class of compounds have served in the development of new flavors, fragrances, medicines and pesticides. While terpenes are made predominantly by plants and microbes, they tend to accumulate in small amounts and as components of complex mixtures. Chemical synthesis of terpenes remains technically challenging, costly and inefficient. Hence, there are many on-going efforts to create robust and efficient biological production platforms for this important class of molecules. The methods described here generate new yeast strains possessing a dispensable mevalonate biosynthetic pathway wherein carbon flux can be diverted to build any chemical class of terpene product desired. First, a genetic screen was developed to select for mutant yeast capable of taking up exogenous sterol under aerobic conditions and eliminating the need for innate ergosterol biosynthesis. This was followed by the introduction of a knockout mutation in the endogenous yeast squalene synthase (ERG9) gene. Thus, yeast lines with a disabled downstream sterol biosynthetic pathway were created and these lines were viable if they were propagated in the presence of an exogenous sterol source. More importantly, these new yeast lines were evaluated for their ability to accumulate high levels of farnesol (FOH), the dephosphorylated form of farnesyl diphosphate (FPP), and a key intermediate in terpene biosynthesis. Theoretically, the flux of carbon to farnesyl diphosphate could be utilized for the biosynthesis of any class of terpene products.

These new yeast lines were derived from the parental strain BY4741. The yeast was first subjected to EMS mutagenesis, followed by selection for growth in the presence of nystatin, squalestatin, and exogenous cholesterol. This screen selected for mutant yeast lines having a dispensable mevalonate pathway and uncharacterized sterol uptake enhancement (SUE) mutations supporting aerobic uptake of exogenous sterol. These mutants were next screened for high-level accumulation of farnesol as an indication of carbon flux to FPP, a key intermediate in terpene metabolism. To further improve the availability of FPP for other biosynthetic designs in these mutants, insertional mutation
into the *ERG9* gene, which encodes for squalene synthase, were created. A series of these yeast strains were then screened for those that accumulated farnesol to levels over 100 mg/L under shake flask culturing conditions. The utility of these new yeast lines for novel terpene production was then qualified and quantified by introducing into the yeast a constitutive expressed *Hyoscyamus muticus* premnaspirodiene synthase (HPS) gene, which encodes for a well characterized sesquiterpene synthase. One of yeast lines, ZX178-08, accumulated premnaspirodiene up to 116 ± 23.6 mg/L with FOH levels of 23.6 ± 14.5 mg/L. In comparison, the parental line BY4741 engineered with the *H. muticus* synthase gene accumulated 10-times less premnaspirodiene, 10.94 ± 3.12 mg/L, with no FOH accumulation detected. Co-expression the HPS gene with a gene encoding for a putative rate-limiting enzyme in the early steps of the mevalonate biosynthetic pathway, an amino-terminal truncated form of the hamster 3-hydroxyl-3-methyl-glutaryl Co-enzyme A reductase gene, improved premnaspirodiene accumulation approximately 50%, up to 170.23 ± 30.44 mg/L.

### 2.2. Background

#### 2.2.1. Terpene physiologic and practical uses

Terpene natural products play important physiological roles in plants, microbes and to some extent in insects, and also have many practical uses as fragrances, flavors, medicines for mankind with tremendous commercial value that have served to attract considerable interest since the 15th century (Buckingham, 2003). Terpenes are a very large class of structurally diverse compounds made in all kingdoms of life (Crowley et al., 1998) and are perhaps the most extensively described class of natural products as evident by well over 100,000 different terpenes reported in the literature. Terpenes are also widely recognized for their diverse utility and applications. For example, many terpenes have been identified on the basis of their activity in herbal medicines, and many such terpenes and their derivatives have been developed into promising and potent pharmaceutical drugs in use today. Artemisinin is a sesquiterpene isolated from the plant Artemisia annua that has been developed as a key pharmacological agent for the control of malaria (Tu, 2011), with annual global demand around 130-150 tons (CHAI, 2009). Likewise, taxol is 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 & Wani, 1995), now widely used to treat patients with lung, ovarian,
breast and brain cancers, and reached annual sales in the US of $1.6 billion in 2000 (Wikipedia).

The structure complexity of terpenes gives them their broad utilities. Menthol is a monoterpene obtained from mint family plants and is a popular ingredient in many foods and consumer products (Bedoukian, 1983). Patchouli, another sesquiterpene, is a popular aromatic found in colognes, perfumes and many other household cleaning products (Wu et al., 2006). The world demand for patchouli is approximately 1200 to 1500 tons/year with a projected increasing demand of 10% -15% per year (Vel & Nugrohowardhani, 2012). Triterpenes such as squalene, obtained from various plant sources and the livers of deep sea sharks, has utility as a nutraceutical product, is used extensively in many types of cosmetics, has special utility as a lubricant for high performance machinery, and is an adjuvant in some pharmaceutical formulations (Bhilwade et al., 2010, Huang et al., 2009, Reddy & Couvreur, 2009).

2.2.2. Terpene production is limited from natural source

Although there is often a high demand for specific terpenes, the actual amount of each terpene from natural sources is limited. Terpene are generally made natively by plants and microbes in small amounts and components of complex mixtures that vary with 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 produced directly from the bark of Pacific yew from late 60’s to early 90’s, and the harvest process killed the trees. Approximately six 100-year old Pacific Yew trees were required to extract enough taxol compound for one cancer patient therapy (Chang & Keasling, 2006). Now paclitaxel, a taxol equivalent, is manufactured with plant cell culture fermentation (Tabata, 2004, Phyton-Biotech). Even though the whole biosynthetic pathway of paclitaxel is not clear, efforts to improve the biosynthesis of intermediates, precursors, and derivatives through metabolic engineering techniques to provide alternative sources to feed the worldwide market demand are the next challenges.

Chemical synthesis of terpenes is often costly, and inefficient (Nicolaou et al., 1994). Chemical synthesis also suffers from generating enantiomeric mixtures, which adds another layer of complications and difficulties for down-stream processing 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 (Keasling, 2009, Martin et al., 2003, Asadollahi et al., 2008, Asadollahi et al., 2010, Kirby et al., 2008, Seki et al., 2008, Fischer et al., 2011, Takahashi et al., 2007). Metabolic engineering and synthetic biology techniques provide powerful tools for developing terpene production platforms. In plants there are two independent terpene metabolic biosynthesis pathways, which increase the opportunities for redirecting metabolic flux to desired high valued products. The MVA (mevalonate) and MEP (methylerythritol-phosphate) pathways both synthesize the basic five carbon building units IPP (isopentenyl diphosphate) and its isomer DMAPP (dimethylallyl diphosphate) necessary for the assembly of all classes of terpenes. The MVA pathway, however, operates in the cytoplasm, while the MEP pathway operates in plastids, which allows for a division of labor for the biosynthesis of specific classes of terpenes and unique opportunities for engineering strategies (Wu et al. 2006). This is in contrast to fungi or bacteria, which possess only one pathway, the MEP pathway in E. coli and the MVA pathway in yeast. *Saccharomyces cerevisiae* is a unicellular eukaryotic organism widely used as a model system to study fundamental aspects of cell physiology, biochemistry and molecular genetics, and is commonly used in fermentations for biotechnology applications. The precursors of all classes of terpene biosynthesis (GPP, FPP, GGPP, and squalene) are natively synthesized in yeast via the resident MVA biosynthetic pathway, which originally suggested that yeast might offer a unique advantage as a production host for terpenes.

2.2.3. Research progress of metabolic engineering sesquiterpene in *S. cerevisiae*

Sesquiterpene are produced upon cyclization or ionization of farnesyl diphosphate (FPP), a 15 carbon intermediate in the MVA pathway. However wild type yeast does not biosynthesis any sesquiterpenes natively. Instead, all the FPP generated in vivo is channeled to the production of sterols (predominately ergosterol), dolichols, ubiquinone and prenylated proteins. Nonetheless, several studies have successfully engineered sesquiterpene production in *S. cerevisiae* based on the manipulation of native genes in sterol biosynthesis pathway, overexpression of a truncated, soluble form of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (tHMGR), selection for a global transcription factor, upc2-1, regulation sterol synthesis, overexpression of the yeast farnesyl diphosphate synthase gene, ERG20, and down-regulation the yeast resident squalene
synthase gene, *ERG9* (Ro et al., 2006). HGMR is a rate-limiting enzyme in sterol biosynthesis, catalyzing the formation of mevalonic acid from HMG-CoA (Coenzyme et al. 1985). *S. cerevisiae* have two functional HMGR genes, HMGR1 and HMGR2. The N-terminus of the encoded HMGR proteins anchors these proteins to the ER (endoplasmic reticulum) membrane, while the C-terminal contains the enzymatic active sites and is oriented for exposure to the cytoplasm (Liscum et al., 1985). These two enzymes are highly regulated by downstream intermediates which impose feedback control and degradation of the HMGR proteins. Over-expression of an N-terminal truncated version of HMGR, leaving the catalytically active C-terminal region, dramatically increased squalene accumulation (Dai et al., 2012, Polakowski et al., 1998). Likewise, a single point mutation in the transcription factor *upc2-1* increased sterol uptake from the culture media 5-fold compared to wild type yeast under aerobic conditions (Crowley et al., 1998), and upregulated several genes in the yeast sterol biosynthetic pathway (Ro et al., 2006). Addition of tHMGR over-expression to these lines also improved amorphadiene production 5-fold. Down regulation of *ERG9* by substituting a suppressible promoter for the native promoter also increased amorphadiene production an additional 2-fold, and when combined with overexpression of *upc2-1* improved the amorphadiene production to 105 mg/L. Adding an extra tHMGR further improved production to 149 mg/L, with overexpression of *ERG20* enhancing production to well above 150 mg/L (Ro et al., 2006). Within these various yeast strains, changing the selection marker from *Leu2* to *Leu2d* (a promoter deletion mutation leaving only 29-bp of the *Leu2* promoter region in the *Leu2* expression cassette) on the expression plasmid also harboring the sesquiterpene synthase, improved plasmid stability and maintained high copy number in *S. cerevisiae*, and subsequently boosted the amorphadiene production to 800 mg/L in non-selection media with shake flask cultures (Ro et al., 2008). Westfalla et al., (2012) reasoned that amorphadiene production in *S. cerevisiae* strain CEN.PK2 could be enhanced because this strain has better characterized physiology and significantly higher sporulation efficiency. Amorphadiene production reached 300 mg/L in CEN.PK2 with similar modifications to strain BY4742, which accumulated 80 mg/L. When extra copies of the *Erg10, 13, 12, 8*, and *IDI* genes were integrated into the genome of CEN.PK2 with galactose inducible promoters to drive high level expression, along with over-expression cassette for the *upc2-1* gene (a gene encoding for a transcription factor effecting expression of the innate mevalonate biosynthetic pathway and anoxia metabolism) and three copies of an HMGR gene encoding for a soluble form of the
enzyme, amorphadiene accumulation increased up to 1200 mg/L in shake flask cultures. A further deletion of the Gal80p gene, the gene encoding for a negative-regulator of galactose regulation of gene expression, from the strain eliminated the need to add expensive sugar galactose for induction of amorphadiene production. Finally the restricted ethanol-pulse feeding and glucose feeding through the fermentation process, resulted in over 40 g/L of amorphadiene accumulating (Westfall et al., 2012). As observed in plants, targeting terpene biosynthesis to non-native compartments within yeast has also proven to enhance terpene accumulation. When Farhi et al. (2011) targeted FPP synthase and either valencene synthase or amorphadiene synthase, two sesquiterpene synthases, to the mitochondria of yeast, sesquiterpene accumulation was improved 8- and 20-fold relative to when the enzymes were directed to the cytoplasm.

Given the already impressive work done to engineer yeast as a terpene production platform, the current effort was directed towards the development of additional yeast strains that arise from a combination of unbiased, conventional genetic screens as well as directed engineering efforts. Our hope was that such an effort would afford yeast strains amendable to further optimization for all classes of terpenes, but initially demonstrated for sesquiterpene production as described here.

2.3. Results

2.3.1. Overall strategy to generate new yeast strains for terpene production

Figure 2.1 outlines the approach used to generate yeast strains providing robust terpene biosynthesis from universal precursors that could 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 cells 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 only take up exogenously supplied sterol from their environment under anaerobic conditions.
In order to be able 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 & 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, and therefore 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 up to the biosynthesis of FPP and hence, 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. 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 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).
Figure 2.1. Overall approach for generating a yeast line that would have a dispensable sterol biosynthetic pathway, which would provide opportunities for diverting intermediates (DMAPP, IPP and FPP) from the mevalonate (MVA) pathway for the biosynthesis of other terpene compounds.
2.3.2. Steps in the development of yeast with a dispensable mevalonate pathway

To generate the new yeast line with high-level FPP pool accumulating suitable for terpene engineering, we subjected the parental wild type yeast line to three phases selection. Phase I selected for aerobic sterol uptake mutants induced by EMS mutagenesis, phase II screened those mutants for those accumulating high levels of FOH, and phase III selected for site-specific erg9 knock out mutants. Figure 2.2 illustrates these 3 phases in the development of the desired yeast line. In phase I, chemical mutagenesis is used to introduce SUE mutations (Bourot & Karst, 1995, Shianna et al., 2001), which are identified by selecting for yeast cells that do not have a functioning ergosterol biosynthetic pathway and can 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 important 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 have 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.

In phase II, yeast lines demonstrating an absolute requirement for exogenous sterols for growth were chemical profiled by GC-MS (Figure 2.3). Aliquots of those yeast lines exhibiting normal growth characteristics, and having growth rates comparable to wild type yeast, were extracted and their chemical constituents separated by gas chromatography and identified by their mass fragmentation patterns. The parental line BY4741 does not accumulate detectable amounts of FOH under these conditions. Eight mutant lines accumulating 50 or more mg/L of FOH (as shown in Figure 2.3) were selected for phase III knockout mutagenesis of the squalene synthase gene, ERG9.
The objective in phase III was to obtain a knockout mutation of the \textit{ERG9} (squalene synthase) gene, thus assuring the dispensable nature of the endogenous mevalonate pathway for ergosterol biosynthesis, thus eliminating the need for the addition of squalestatin to the culture medium. Site specific recombination was afforded by appending 5’ and 3’ regions surrounding the native \textit{ERG9} gene onto a hygromycin selection marker gene (see supplementary materials and methods information), then introducing this linear gene construct into selected yeast lines from the phase II screening under conditions to promote site-specific, double recombination with the native \textit{ERR9} gene. The knockout mutants were then selected by plating the cells in the presence of ergosterol and hygromycin. Recombination as depicted in Figure 2.2 should result in the coding region of the \textit{ERG9} gene being displaced or substituted by the hygromycin resistance marker gene. Replica of the obtained single colonies on plates plus and minus ergosterol revealed positive \textit{erg9} knockout colonies can only grow on plates with sterol supplementation, but cannot grow on plates without sterol supplementation. Further confirmation of such a substituted \textit{erg9} knockout event 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 \textit{ERG9} coding region. Using genomic DNA isolated from hygromycin resistant colonies as template with a hygromycin specific primer (HphF) and a primer specific to a genomic DNA sequence found 3’ to the \textit{ERG9} gene (ERG9 450DwR), a PCR amplification product of approximately 1,538 bp would be expected and is evident in the colonies so tested in Figure 2.4 A.

Independent colonies from each of the \textit{erg9} knockout lines were then re-evaluated for their growth in liquid media and the dispensable nature of their mevalonate pathway by checking for their accumulation of FOH (Figure 2.5). These new yeast lines with \textit{erg9} knockout and dispensable sterol pathway accumulate significant amount FOH, the dephosphorylated form of farnesyl diphosphate, in comparison with parental yeast line BY4741, which have no detectable level of farnesol (Figure 2.4 B). Nine yeast lines accumulating more than 60 mg/L farnesol in test tube scale culture were selected for qualification of terpene production capacity.
**Figure 2.2.** Generation of new yeast strains for terpene production.
A) Three phases in selection of yeast lines dependent upon exogenous sterols for aerobic growth and having a dispensable mevalonate biosynthetic pathway. B) Steps in the development of yeast having a dispensable mevalonate biosynthetic pathway and the number of colonies screened at each stage.
Figure 2.3. Quantitation of FOH levels in yeast lines demonstrated to have an exogenous sterol requirement for growth.

Yeast lines were grown as test tube shake cultures with 3 ml of YPD media containing 40 µg/ml of ergosterol and 40 µg/ml of squalestatin for 6 days prior to sampling the cultures. One ml aliquots of cultures were mixed vigorously with 1 ml of acetone, then allowed to stand for 15 min. One ml of hexane containing a cedrene external standard was then added, vortexed, centrifuged in a clinical centrifuge for 5 min, and the upper hexane phase removed and concentrated to 100 µl under a nitrogen stream. One µl
 aliquots of the hexane extracts were then subjected to GC-MS and FOH levels quantified relative to the external standard.
Figure 2.4. PCR and metabolites confirmation of erg9 knockout mutation.

A) PCR confirmation for the erg9 knockout mutation. DNA isolated from 4 independent colonies selected for substitution of the hygromycin resistance gene for the ERG9 gene was used as PCR template with a hygromycin specific primer and a primer specific for the genomic DNA surrounding the ERG9 locus. If the HPH gene did insert and replace the ERG9 gene, the expected amplification product would be 1,538 bp. B) Metabolite confirmation of erg9 knock out mutation. FOH accumulation in a yeast line (178-08) selected for a dispensable mevalonate biosynthetic pathway plus erg9 knock out in comparison to that accumulating in the parental line (BY4741) used to generate the new mutant yeast lines. GC-MS chromatograph of hexane extracts prepared from the wild type and engineered yeast lines. No farnesol accumulated in parental yeast BY4741. Over 100 mg of FOH/L accumulated in the newly developed yeast line ZX178-08, as quantified on the basis of a cedrene external standard.
Figure 2.5. Quantitation of FOH levels in *sue, erg9* mutant lines of yeast demonstrated to have an exogenous sterol requirement for growth and resistance to hygromycin.

Cultures were grown in 3 ml test tube cultures of SCE media supplemented with histidine, leucine, uracil, tryptophan and methionine for 6 days before extracting and quantifying their FOH levels by GC-MS as described in Figure 2.3.
2.3.3. Qualification of a new mutant yeast strain for its utility to produce a desired terpene compound

Nine of the yeast lines harboring a SUE mutation and having the native ERG9 gene deleted were evaluated indirectly for the available of terpene biosynthetic intermediates, and specifically FPP, to support sesquiterpene biosynthesis in comparison to the parental strain BY4741 (Figure 2.6). Hyoscyamus prennaaspirodiene 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 prennsapirodiene 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 prennsapirodiene, 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 prennsapirodiene, 10.94 ± 3.12 mg/L, with no farnesol accumulation detected. The control line ZX178-08 without HPS gene expression produced farnesol 101.36 ± 16.84 mg/L, which was less than the prennsapirodiene plus FOH (~140 mg/L) produced in ZX178-08 strain expressing the HPS gene. This indicated that the production of terpene in this new yeast line is related with the specific catalytic terpene synthase activity and, because the FPP is known to act as a feedback signal that controls ubiquitination and therefore degradation of HMGR2 (Gardner & Hampton, 1999, Hampton & Bhakta, 1997). HMGR is a putative rate-limiting step control carbon flux in the mevalonate pathway (Coenzyme et al. 1985), the FPP level could also be self regulating. When part of the FPP pool is diverted to a sesquiterpene production, more FPP is produced, which might explain how Zx178-08 with HPS produces more total FPP than ZX178-08 alone.

HMGR catalyzes a key regulatory step controlling sterol biosynthesis metabolism, the reaction of conversion of 3-hydroxy-3-methylglutaryl coenzyme A to mevalonate. In addition to being subject to regulation at the protein turnover level as noted above, the 5' end of HMG CoA Reductase gene also contains sequences responsible for sterol-mediated inhibition of transcription (Osborne et al., 1985). Hence, to engineer elevated HMGR enzyme activity into yeast and further enhance terpene production, a truncated and full-length version of the hamster HMGC0A reductase were co-expressed with HPS.
gene in the new developed yeast line ZX178-08. The truncated hamster HMGR (tHMGR) was produced by removing the regulatory and N-terminal, membrane-spanning domains, which was previously shown when constitutively expressed in tobacco led to a 3- to 6-fold increase in the total HMGR enzyme activity and a 3- to 10-fold increase in the total sterol accumulation (Chappell et al., 1995). Co-expression HPS gene plus full length HMGR gene, resulted in improved sesquiterpene production to 132±11.5 mg/L with 31.21±7.2 mg/L FOH. When the HPS gene and tHMGR gene were co-expressed, premnaspirodiene production further improved to 170.23±30.44 mg/L with 21.88±3.82 mg/L farnesol. Figure 2.6 B and C compare cell culture growth and premnaspirodiene production by yeast strains developed for enhanced levels of FPP plus truncated HMGR (Figure 2.6 B) or full length HMGR (Figure 2.6 C) over-expression over 12 days time course.
HPS+tHMGR in ZX17808

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mg/L

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Day

0 2 4 6 8 10 12 14

FOH

Prem

OD
Each of the ZX cell lines as well as the wild type parental line (BY4741) were independently transformed with an expression vector harboring the *Hyoscyamus* premnaspirodiene synthase gene or co-expression along with truncated hamster HMGR or full length (intact) HMGR. The yeast lines were then grown for 12 days prior to chemically profiling for their cell constituents by GC-MS and quantifying the levels of premnaspirodiene and farnesol found in each culture. (A). Comparison of cell culture growth and premnaspirodiene production by yeast strains developed for enhanced levels of FPP plus truncated HMGR (B) or full length HMGR (C) over-expression over 12 days time course.

Figure 2.6. Comparison of terpene accumulation levels in yeast lines (ZX series) developed as terpene production platforms.
2.4. Discussion

Terpenes are a highly diverse class of natural products with important biological activities and commercially value. Nonetheless, the development of single entity terpene products has been hampered by the high cost and low efficiency of extraction from its natural sources, and the complexities associated with their chemical synthesis. Many labs have focused on taking advantage of the native sterol biosynthesis pathway in yeast for the development of a terpene production platform (Asadollahi et al., 2008, Martin et al., 2001, Ro et al., 2008, Ro et al., 2006, Takahashi et al., 2007, Westfall et al., 2012). Yeast, as a fermentable organism, lends itself to the notion of building a cell factory for production of stereochemically distinct terpenes without limitations of availability.

In this present study, we first developed new yeast strains with a dispensible sterol biosynthetic pathway. In phase I, chemical mutagenesis was used to isolate SUE mutations to obtain yeast that could take up exogenously supplied sterol from the medium under aerobic condition. In phase II, high FOH accumulating lines were identified via a GS-MS screen when the individual lines were grown in the presence of squalestatin, an inhibitor of squalene synthase. Because FOH is derived from the dephosphorylation of FPP, these high FOH accumulating lines were inferred to have high levels of FPP accumulation. Since nearly all of the FPP is normally used for the biosynthesis of sterols, blocking the flux of FPP to sterols would conceptually increase the accessible FPP, as well as earlier precursors, for other enzymes redirecting carbon flux to the building of other desired end products. Subsequently in phase III, an erg9 (yeast squalene synthase) knock out mutation was introduced, replacing the need for using exogenous squalestatin to inhibit squalene synthase.

If our engineering design was correct, then we would expect enhanced levels of FPP to be available for sesquiterpene biosynthesis. We chose to test this possibility by over-expressing the gene encoding for premnaspirodiene synthase, HPS, a robust sesquiterpene synthase. Premnaspirodiene accumulation in the selected yeast line ZX178-08 was approximately 10-fold greater than when the HPS gene was expressed in the original yeast strain BY4741. We also evaluated if it were possible to push more carbon down the mevalonate pathway to FPP by over-expressing the putative rate-limiting enzyme HMGR (Coenzyme et al. 1985). When a truncated form of the hamster HMGR enzyme was expressed from a high copy plasmid under the control of the strong
constitutive GPD promoter, a further increase in premnaspirodiene accumulation of 50% in shake flask cultures was observed. The increased premnaspirodiene production levels are two-fold higher than our previous efforts with yeast lines having a different genetic background (Takahashi et al., 2007). The current results suggest that we might be able to make further improvements in these yeast lines in terms of terpene production by integrating the truncated HMGR gene in multiple copies across the yeast genome, perhaps targeted to rDNA loci or Ty repetitive elements (Romanos et al., 2004). Such a line would have the advantage of not having to be grown under selection pressure for an autonomous plasmid, which could represent a significant cost savings as well as result in better growth characteristics of the yeast cultures.

Engineering yeast for sesquiterpene production has also been an objective of the Keasling research group at the University of California, Berkeley, targeting a means for the biosynthesis and accumulation of artemisinin, a therapeutic reagent for the treatment of malaria. The basic strategy of this group is summarized in Figure 2.7 A and consists of the following modifications: 1) to limit carbon flux to downstream biosynthetic processes, the native squalene synthase gene promoter was replaced with a methionine suppressible promoter, pMet3; 2) To increase the upstream carbon flux to FPP biosynthesis, high level over-expression of the ERG10, 13, 12, 8, 20, tHMGR, and IDI genes were achieved by either using conventional over-expression gene cassettes or by up-regulating the UPC-2 transcription factor (green highlighted genes are up-regulated by upc2-1); 3) To further increase the pool of isoprenoid intermediates that could be channeled to sesquiterpene production, a defective leu-2d selection marker was used to increase the copy number of the corresponding plasmid with hopes this would translate into additional copies of the associated terpene biosynthetic genes being expressed; and 4) Optimization of medium components leading to enhanced growth and fermentation production of sesquiterpenes (Ro et al., 2008, Ro et al., 2006, Westfall et al., 2012).

The greatest difference between the yeast lines developed here in comparison to the amorphadiene production lines developed by the Keasling group is the generation of the dispensable sterol biosynthetic pathway and the complete shut down of carbon flux to the downstream sterol biosynthetic pathway by knocking out the ERG9 gene as shown in Figure. 2.7 B. In the yeast engineered for amorphadiene accumulation, the native ERG9 promoter was replaced with the MET3 promoter, which allowed for the
suppression of squalene synthase gene expression by exogenous application of methionine at the late stages of culture growth. Moreover, the methionine suppression mechanism is dependent on the methionine concentration and the metabolic state of the cell culture. Yeast consume methionine, so expression of the ERG9 gene can occur if the methionine levels drop below a key threshold level. Accurate monitoring of the methionine concentration and the ability to readily add methionine are essential for this control mechanism of squalene synthase. In contrast, the ZX yeast strains described here contain a complete knockout of the ERG9 gene, hence a relatively large and accessible pool of FPP and earlier MEP intermediates are available for diversion to the biosynthesis of desired sesquiterpenes or other terpene classes. The new yeast line ZX178-08 line, which co-expresses a tHMGR in combination with the sesquiterpene synthase gene, HPS, accumulated 170.23±30.44 mg of premnaspirodiene/L, in comparison to EPY225 from Keasling lab, which has the equivalent modification but methionine suppression strategy to inhibit squalene synthase instead of complete knockout, produced only 44 mg amorphadiene/L. Although the specific catalytic activities of sesquiterpene synthase will effect the sesquiterpene production, our newly developed yeast lines also show much higher FPP baseline levels than EPY225, which suggests that with additional efforts as described in Fig2.7A our newly developed yeast terpene production platform has the potential to produce more terpene compounds and further improve the state of art of metabolic engineering of terpene production in yeast.

All the genes engineered into the ZX yeast lines are driven by a strong constitutive promoter, the pGPD promoter, which natively controls expression of the glyceraldehyde-3-phosphate dehydrogenase gene (Janke et al., 2004). This contrasts with the galactose inducible promoter used in constructing the yeast lines illustrated in Figure 2.7A, which requires the addition of a relatively expensive inducer (galactose) at the right stage of culture development in order to affect the appropriate timing of expression of the downstream genes. Efficiency of the pGal promoters is also strongly suppressed by the glucose in the culture medium. Hence, one must wait for all the glucose to be utilized by the culture before inducing the cultures with galactose. This becomes even more demanding because the depletion of glucose results in metabolic shifts in the cultures, the so-call Pasteur effect, wherein the cells go into a state of anaerobic catabolism, a state that is not necessarily conducive to anabolic biosynthetic reactions. The ZX178-08 line would not suffer such a shift because they can be grown in fed-batch conditions to
maintain a constant glucose supply that supports the highest rates of cell proliferation and aerobic metabolism.

In summary, new yeast strains have been developed using novel selection mechanisms for genetic and molecular genetic mutants engineered for a dispensable pathway that can be partitioned to the biosynthesis of terpenes of choice.
Figure 2.7. The mevalonate (MVA) pathway in yeast and the efforts made for utilizing this pathway for sesquiterpene production by the Keasling group (A) and those developed in the current work (B).

Genes directly over expressed by extra copies integrated into yeast chromosome or on plasmid are in purple letters; genes up-regulated by over expression of the transcript factor upc2-1 are highlighted by green boxes; terpene production in blue bars and orange bars represent culture growth in selection media or rich non-selection media, respectively. All terpene production titers presented here were in shake flask scale culture. The greatest advantage of the work reported here in terms of sesquiterpene production is the complete knockout of squalene synthase, which necessarily stops carbon flux to sterol biosynthesis, yet avails the accumulation of FPP to sesquiterpene biosynthesis (B).
2.5. Materials and Methods

2.5.1. Chemical and media preparations

All chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO), BD Bioscience (Franklin Lakes, NJ), or Fisher Scientific (Chicago, IL), while reagents for molecular manipulations were purchased 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. YPD media for growing yeast without selection consisted of 1% Bactoyeast extract, 2% Bacto-peptone, and 2% glucose. YPDE media was YPD media supplemented with ergosterol (40 mg/L) for ergosterol dependent lines. YPDNCS media for the SUE mutation screening was YPD media supplement with 40 mg/L Nystatin, 40 mg/L cholesterol and 40 mg/L squalestatin. YPDSE media was YPD media supplement with 40 mg/L squalestatin and 40 mg/L ergosterol. Minimal media, SCE (pH 5.3), contained 0.67% Bacto-yeast nitrogen base (without amino acids), 2% dextrose, 0.6% succinic acid, 0.14% Sigma yeast dropout solution (-his,-leu,-ura,-trp), uracil (300 mg/L), L-tryptophan (150 mg/L), L-histidine (250 mg/L), L-methionine (200 mg/L), L-leucine (1g/L) and 40 mg/L ergosterol. Cholesterol and ergostrol stocks were 10 mg/mL in 50% Triton X-100, 50% ethanol and kept at -20˚C. Selection media was prepared similarly except without supplementing the media with the indicated reagent based on the yeast auxotrophic makers. All solid media plates were prepared with 2% Bacto-Agar.

2.5.2. Ethyl methane sulfonate (EMS) mutagenesis

Strain BY4741 (MATa;his3Δ1;leu2Δ0;met15Δ0;ura3Δ0) (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 OD600 reached approximately 1.0, the cells were spun down by centrifugation (10 min at 4,000 xg), and washed twice with 20 ml 0.1M sodium phosphate buffer, pH7.0. Cells were concentrated by centrifugation again, re-suspended in 1 ml 0.1M sodium phosphate buffer, transferred to a 14 ml FALCON culture tubes, treated with 300 µl EMS (1.2g/ml, Sigma), followed by incubation at 30°C for 1 hour with shaking. To stop the mutagenesis, 8 ml of sterile 5% sodium thiosulfate (Fisher) were added to yeast cells to inactive 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 50mg/L cholesterol, 50mg/L nystatin, 50 mg/L squalesatin, 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.

2.5.3. Yeast transformation and culture performance

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. About 1 µg of plasmid or about 5 µg of linearized DNA was used per transformation and followed by selection on agar plates of SCE medium lacking specified amino acids for the auxotrophic markers or YPDE containing 300 mg/L hygromycin B for screening for erg9 knockout at 30°C. Variable numbers of independent colonies were subsequently picked and used to start 3 ml cultures in minimal media to characterize their terpene production capacities. Aliquots of these cultures were analyzed for terpene production after 6 days of incubation at 30°C with shaking by GC-MS. 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 30 mL shake flask cultures. Starter cultures grown to saturation in minimal media were inoculated into 30 ml SCE media and 1 mL aliquots withdrawn at every other day intervals for 10-15 days. Cell growth was monitored as the change in optical density at 600 nm every two days, using appropriate dilutions for cultures at later stages of growth. Terpene production was determined by GC-MS similar to the initial screening method.

2.5.4. GC-MS detection and quantification of terpenes

To determine terpene accumulation levels, aliquots of cultures grown for 6 to 12 days were extracted with hexane and aliquots evaluated by GC-MS. In general, to 1 volume of culture, 1 volume of acetone was added and mixed vigorously for 3 to 5 min to lyse the cells. The sample was then allowed to incubate at room temperature for 10 min before adding 1 volume of hexane containing a known amount of cedrene external standard. The mixture was again mixed vigorously, then centrifuged in a clinical centrifuge for 5 min at maximum speed. The upper organic layer was collected and when necessary,
concentrated under a N2 stream to 1/10 the original volume. An aliquot of the organic phase (1 μl) was then analyzed by GC-MS with a Varian CP-3800 GC coupled to a Varian Saturn 2200 MS/MS (Varian Medical Systems) using a Supelco SLB-5ms fused silica capillary column (30 m x 0.25 mm x 0.25 μm film thickness, Supelco). The initial oven temperature was set at 70 °C for 1 min, ramped to 200 °C at 8 °C/min, and then ramped to 300 °C at 20°C/min and held for 5 min more. Farnesol and premnaspirodiene levels were calculated relative to the cedrene external standard.

2.5.5. Construction of the squalene synthase (ERG9) Knockout mutation

The primers ERG9PS1 and ERG-250downS2 were used to amplify the hygromycin resistance gene, hphNT1, from the PFA6-hph-NT1 vector (Janke et al., 2004), and at the same time add DNA sequences homologous to regions surrounding the ERG9 gene in the yeast genome. These primers are flanked by 42 nucleotide sequences (underlined) homologous to DNA sequences found 250 base pairs 5’ (upstream) and 3’ (downstream), respectively, of the ERG9 gene found in the yeast genome. The purified PCR fragment was transformed into various yeast lines identified for their ability to accumulate farnesol (Figure 2.3) and grown in 2 ml of YPDE media for an additional 6 hours before being plated on YPDE hygromycin (300mg/L) agar plates at 28°C. Independent single colonies were picked for ergosterol dependent test, PCR confirmation of recombination with hphF and ERG9 450DWR primer, as well as farnesol production analysis. The recombination sequence was further confirmed by DNA sequencing of the corresponding PCR amplification product.

2.5.6. Expression of the HPS and HMGR genes in yeast

The yeast GPD promoter (Pgpd) was amplified from the PYM-N14 plasmid described by using the primers GPD-BamHIF and GPD-NotIR primers and inserted into the pESC-His vector digested with BamHI and NotI to replace the original GAL1/10 promoters. The resulting plasmid was named pESC-His-gpd. The HPS gene was cloned into NotI and SpeI sites of pESC-His-gpd to obtain the yeast expression vector pESC-His-gpd-HPS as previously by Takahashi et al. (2007). pESC-Leu-gpd vector was built as the same way as pESC-HIS-gpd vector. HMGR full and tHMGR were cloned into NotI and SpeI sites of pESC-LEU-gpd to obtain the yeast expression vector pESC-LEU-GPD-tHMGR and pESC-GPD-HMGRfull constructs. Yeast lines transformed with this construct were then evaluated for their production of the sesquiterpene premnaspirodiene as a measure of
the available of intermediates of the mevalonate biosynthetic pathway for the biosynthesis of new terpenes.
Figure 2.8. Constructs used for evaluate yeast sesquiterpene productions.

Yeast expression vector designed for the strong, constitutive expression of the sesquiterpene synthase HPS, tHMGR, HMGR genes directed by the gpd promoter (Pgpd) and termination provided the ADH terminator sequence (T-ADH) with auxotrophic marker His or LEU.
Table 2.1. Primers used for the various molecular manipulations describe in materials and methods.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERG9pS1</td>
<td>GTACATTTCATAGCCCATCTTTCAACAAACAATACCGACTTACCCGTACGCTGAGGTGAC</td>
</tr>
<tr>
<td>ERG9 250dwS2</td>
<td>CAGATTGACGGAGAGGGCCACATTGGTTGTCTGGCAATTTAATCAGTTTCAGGTGCTC</td>
</tr>
<tr>
<td>Hph F</td>
<td>ATGGGTAAGACCGCTGAACCTCA</td>
</tr>
<tr>
<td>Hph R</td>
<td>TTATCTCTTTGCGCTCCGACGAG</td>
</tr>
<tr>
<td>ERG9 450dwR</td>
<td>AGATGCTAGTGAATGGCAGAG</td>
</tr>
<tr>
<td>ERG9p300upF</td>
<td>TGCTTACACACAGATCAGACGAGCTGC</td>
</tr>
<tr>
<td>ERG9 300R</td>
<td>CTCGTGGAAGGTGACGCAAC</td>
</tr>
<tr>
<td>HPS NolI F</td>
<td>GGGGCGCCGCGCAAAAACATGGGCCCCAGCTATAGTGATGAG</td>
</tr>
<tr>
<td>HPS SpeIR</td>
<td>GACTAGTTCAAATATCAATAGAATCCACC</td>
</tr>
<tr>
<td>pGPD-BamHI F</td>
<td>CGGGATCCAGTTTATCATTATCAATAGCTCGCC</td>
</tr>
<tr>
<td>pGPD-NotlR</td>
<td>GGGGCGCCGCGACGCTAGTTTATCAATAGCTCGCC</td>
</tr>
<tr>
<td>tHMGR-NotlF</td>
<td>GGGGCGGCGCCGCAAAAACATGTTTGTCAGACTTTTCCGTATGC</td>
</tr>
<tr>
<td>tHMGR-SpeIR</td>
<td>GACTAGT TCAAGCTGACTTTCTTGTGCACGTTCTTG</td>
</tr>
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Chapter 3: Engineer diterpene production in yeast

3.1. Summary

To more fully demonstrate the utility of yeast line ZX178-08 as a general production platform for diverse terpenes, the ability of this line to generate diterpenes was next investigated. Diterpenes are used for a variety of industrial manufacturing needs, serve as hormones or growth regulators in plants, possess anti-oxidant activities, which lead to their use in human nutraceuticals, and have found significant applications in pharmaceutical drug development. The intent of engineering ZX 178-08 for diterpene biosynthesis was to document the easy of engineering this yeast for the efficient diversion of the isoprenoid pathway intermediates to the biosynthesis of another class of terpene compounds and to identify the molecular conditions for high-yield production of diterpene hydrocarbons and hydroxylated derivatives generating additional high-valued chemical entities. When expression of the diterpene synthase gene encoding for abietadiene synthase (ABS) was coupled with the co-expression of a mutant avian farnesyl diphosphate synthase (mFPS) that exhibits a preferred biosynthesis for geranylgeranyl diphosphate (GGPP) rather than FPP, a dramatic accumulation of abietadiene at the expense of farnesol accumulation was noted in comparison to just expression of the ABS gene by itself. A variety of gene expression promoter combinations were also examined. The strategy for this effort varied the gene promoter element driving expression of the mutant FPS gene on one plasmid construct in combination with the various promoters also directing expression of a fungal kaurene synthase (FKS, another diterpene synthase) gene. The best promoter combination set for diterpene hydrocarbon production was determined to be use of the glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter for both heterologous genes.

Optimization of medium and growth conditions also was shown to dramatically influence overall diterpene accumulation. Using a 5.5 L fed bath fermentation system, about 569 mg/L kaurene and approximately 207 mg/L abietadiene plus 136 mg/L additional diterpene isomers were achieved. To engineer more highly modified diterpenes and especially those molecules that might have greater industrial, agricultural or medicinal applications, co-expression of four genes, mutant FPS (FPP synthase), FKS (kaurene synthase), FKO (fungal kaurene oxidase) and CPR (Cytochrome p450 reductase), all
driven by GPD promoters in various combinations with 3 plasmid vectors, kaurenoic acid production reached 514 mg/L and byproduct kaurene and kaurenal at 71.7 mg/L and 20.1 mg/L, respectively, in fed batch fermentation conditions.

3.2. Background

3.2.1. Diterpene applications and uses

Diterpenes are a class of compounds within the much larger terpene family of molecules (Fig. 3.1). Terpenes, in general, are built upon a 5 carbon-repeating unit giving rise to classes of compounds having 10 (monoterpenes), 15 (sesquiterpenes), 20 (diterpenes), and more carbons. Diterpenes are known to have diverse biological and practical applications. In plants, specific diterpenes serve as hormones or growth regulators (i.e. gibberelic acid derivatives) (Yamaguchi, 2008) while others serve as accessory photopigments funneling energy from light capture to the light reactions of photosynthesis (Havaux et al., 2004). Other diterpenes provide protection against oxidative radicals (Grassmann, 2005). The anti-oxidant activity of diterpenes has also led to their use in human nutraceuticals and medical applications (Cardenas et al., 2011). Perhaps the most widely recognized diterpene is taxol, used very successfully and extensively for the treatment of a variety of cancers (Wall & Wani, 1995). Specific diterpenes have also found use in the control of dental caries providing antimicrobial activities (Porto et al., 2009). Other diterpenes have found utility in chemical manufacturing (Bremser et al., 2006).

3.2.2. Diterpene engineering in microbial host cells

Diterpenes are traditionally obtained from plant sources. However, they are often found in only small amounts and as components of complex mixtures that vary with growth and environmental conditions, making it difficult to obtain large amounts of any one diterpene constituent (Wu et al., 2006). Chemical synthesis of diterpenes is often costly and inefficient (Nicolaou et al., 1994). Chemical synthesis also suffers from generating enantiomeric mixtures, which adds other complications if one particular stereochemical form of a terpene are desired. Given such difficulties, there are many on-going efforts to create robust, reliable and efficient biological systems for the production of distinct diterpenes, and more so for the generation of stereochemically pure forms of diterpenes (Anterola et al., 2009, Engels et al., 2008, Kovacs et al., 2007, Roberts, 2007, Dejong et
Taxadiene, a diterpene derivative, is the key intermediate of paclitaxel, which is widely used in cancer chemotherapy. Metabolic engineering of taxadiene has resulted in 1.3 mg/L in *E. coli* (Huang et al., 2001) and 1 mg/L in yeast (Dejong et al., 2006). Recently, advanced improvements in taxadiene engineering achieved 1g/L in *E. coli* (Ajikumar et al., 2010) under fedbatch fermentation through a multivariate-modular approach for optimize balancing and channeling the overflow flux from IPP and DMAPP toward taxadiene biosynthesis. Recent approaches for improving diterpene production in heterologous hosts have included over-expression of fusion enzymes to increase the association of successively acting enzymes and thus channeling of intermediates towards the biosynthesis of desired end-products. One example of this strategy was the observation of 238.8 mg geranylgeraniol/L by yeast over-expressing several diterpene biosynthetic enzymes (Tokuhiro et al., 2009). Using a similar strategy for mitiradiene biosynthesis, the diterpene precursor to the Chinese herb medicine tanshionones, up to ~480 mg/L were achieved in optimized yeast fermentation (Zhou et al., 2012, Dai et al., 2012).

### 3.2.3. Overall strategy of engineering diterpene in our yeast

Figure 3.2 outlines the approach we used to generate yeast lines that provide for the robust 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.

In order to be able to efficiently channel terpene biosynthetic intermediates from the ergosterol biosynthetic pathway (Fig. 3.2A), a SUE (sterol uptake enhancement) mutation supporting the aerobic uptake and utilization of exogenous sterol was first created (Bourot & Karst, 1995, Shianna et al., 2001). A SUE mutant is thus a yeast line that can meet all its sterol needs by an exogenous source of sterol, and therefore
making the endogenous ergosterol biosynthetic pathway dispensable. The SUE mutation was then complemented by the introduction of a knockout mutation in the \textit{ERG9} gene (squalene synthase) (Zhang et al., 1993), resulting in a yeast line where the MVA pathway was still operational up to the biosynthesis of FPP and hence, intermediates in the pathway (DMAPP, IPP and FPP) could be diverted to the biosynthesis of other non-essential terpene components.

The intent of the current study in Chapter 3 was to further this technology for the efficient diversion of isoprenoid pathway intermediates to the biosynthesis of diterpenes, to identify the molecular means for high-yielding conditions for the production of diterpene hydrocarbons and decorating the diterpene scaffolds to generate additional high-valued chemical entities.

Yeast line ZX178-08 was previous developed (see Chapter 2), having a dispensable sterol biosynthetic pathway and accumulating high levels of FPP, but limited GGPP as indicated by no detectable accumulation of geranylgeraniol. The current chapter describes the generation of yeast lines that can be utilized for the production of diverse and high-valued diterpenes through heterologous expression of a novel GGPP synthase and diterpene synthases, along with diterpene oxidase for further decoration.
Figure 3.1. The representative compounds of each class terpenoids and the prenyl diphosphates they are derived from.

All terpenes are derived from allylic diphosphates, which are polymers of repeating isopentyl units (IPP) put together by the action of prenyltransferases. Monoterpenes are derived from the C10 precursor geranyl diphosphate (GPP), sesquiterpenes from farnesyl diphosphate (FPP), and diterpenes from geranylgeranyldiphosphate (GGPP) by the action of terpene synthases. The terpene synthases impose unique regio- and stereo-chemical configurations onto the reaction intermediates during catalysis that impart novel characteristics to the reaction products.
Figure 3.2. The strategies for develop a yeast line suitable for engineering diterpene chemicals.

Schematic diagram of the overall approach for generating a yeast line that would have a dispensable sterol biosynthetic pathway (panel A), which would provide opportunities for diverting intermediates (DMAPP, IPP and FPP) from the mevalonate (MVA) pathway for the biosynthesis of diterpene compounds (panel B).
3.2.4. Building a GGPP pool in yeast by overexpression of a mutant avian FPS synthase

The limited accumulation of geranylgeraniol in our previous developed yeast line ZX178-08 indicated the necessity of co-expression of a GGPP synthase (a prenyltransferase) to generate a GGPP pool sufficient for its diversion to diterpene biosynthesis. Prenyltransferases catalyze the condensation of allylic diphosphates to build increasing polymeric forms of allylic diphosphates. For example, trans-prenyltransferase synthesize products up to C50 in chain lengths and can be divided into short chain (C10-C25), medium chain (C30-C35), and long-chain (C40-C50) prenyltransferases (Chang et al., 2006). GPS (geranyl diphosphate synthase C10), FPS (farnesyl diphosphate synthase, C15), GGPPS (Geranylgeranyl diphosphate synthase, C20) belong to the short chain trans-prenyltransferases, and they all share a significant level of amino acid sequence similarity. The sequence alignments include five highly conserved sequence domains, which include two important Asp-rich motifs, FARM (first Asp-Rich motif) and SARM (second Asp-rich motif), which share the consensus sequence DDX2-4D, X representing any amino acid (Bouvier et al., 2005). These two highly conserved Asp-rich motifs are important for catalytic function and substrate binding. For short-chain trans-prenyltransferases, a bulky amino acid residue generally occupies the fourth or fifth position upstream from the FARM. FARM has been designated as the chain length determination region (Bouvier et al., 2005, Narita et al., 1999). SARM provides a Mg$^{2+}$ binding site and is important for overall prenyl product formation (Bouvier et al., 2005). Both FARM and SARM are important for enzyme catalytic activity and product chain determination. Data based on the FPS 3D crystallographic structure and site direct mutagenesis, mutant forms of FPS showed that the amino residue at the fourth and fifth position before the FARM block the allylic substrate site and prevent further product chain elongation (Bouvier et al., 2005). Directed mutagenesis of FPP synthase can generate mutant enzymes capable of producing shorter chain product GPP or longer chain prenyl diphosphates. For example, mutant avian farnesyl diphosphate synthase (FPS) has the capability of producing geranyl diphosphate (A116W, N144W) (Fernandez et al., 2000), geranylgeranyl diphosphate (F112A), geranylfarnesyl (C25) diphosphate (F113S), and longer chain prenyl diphosphates (F112A/F113S) (Tarshis et al., 1996). Equally important is that the relatively activity of avian mutant enzymes, at least those that can produce longer prenyl diphosphate products than FPP, retain about the same
level of catalytic activity as the wide type avian FPS (Tarshis et al., 1996). Such mutants could give us additional tools for engineering diterpene metabolism in yeast.

GGPP is precursor for diterpene, carotenoids, chlorophylls, geranylgeranylated proteins, which is an essential post-translational modification for signaling proteins such as Ras, Rho, Rab, and Rac (Takai et al., 2001). Neither native (BY4741) nor our modified yeast ZX series lines accumulate GGOH (geranylgeraniol), indicating the limited availability of GGPP in these lines. In other work from Wu et al. (2006), co-expression of a prenyltransferase, capable of supplying the appropriate allylic diphosphate substrate for terpene synthase, along with an appropriate terpene synthase was sufficient to enhance overall terpene accumulation more than 1,000 times higher than if only the synthase were expressed by itself. We therefore reasoned that expression of a mutant form of the avian FPS (substituting phenylalanine 112 for alanine) in yeast line ZX178-08 might result in the build-up of a GGPP pool. And, if we were to co-express a suitably robust diterpene synthase, then this pool of GGPP might be diverted to the biosynthesis and accumulation of a diterpene in the yeast culture.

3.2.5. Kaurene and abietadiene synthase and the enzymes responsible for converting these hydrocarbons to oxidized forms

Ent-kaurene is an important precursor to gibberellins (GAs), diterpene phytohormones which regulate various aspects of plant developments including shoot and stem elongation, germination, dormancy, flowering, and leaf and fruit senescence (Hedden & Phillips, 2000). In higher plants, ent-kaurene is formed by the successive action of two cyclase enzymes, CPP synthase and ent-kaurene synthase, which catalyze the cyclization of GGPP to CPP (copalyl diphosphate), followed by the cyclization of CPP to kaurene as shown in Fig. 3.3 (Keeling et al., 2010). In contrast, fungal ent-kaurene synthase is a single bifunctional enzyme that catalyzes the two-step conversion of GGPP to ent-kaurene (Toyomasu et al., 2000). Gibberella fujikuroi is the rice pathogenic fungus causing lodging of field grown plants by producing large quantities of GAs, which result in abnormal growth characteristics of plants leading to the lodging phenotype. The fungal enzymes responsible for gibberellin biosynthesis have been extensively studied (Toyomasu et al., 2000). In considering engineering diterpene biosynthesis into yeast, the advantage of having a single, bifunctional enzyme that can catalyze the two-step cyclization reactions represents a distinct advantage to attempting the co-expression of...
two successively acting enzyme activities. In fact, an attempt to over-express a codon optimized form of the *Gibberella fujikuroi* kaurene synthase (FKS) in yeast was reported by Toyomasu et al. (2000).

The *Arabidopsis* ent-kaurene oxidase gene (AtKO1), belonging to a cytochrome P450 monooxygenase subfamily CYP701A, was first isolated by Helliwell et al. (Helliwell et al., 1998), and found to catalyze the three step oxidation ent-kaurene to ent-kaurenoic acid when expressed in yeast (Helliwell et al., 1999). Ashman et al. (1990) characterized an ent-kaurene oxidase activity associated with the crude cell extracts from *Gibberella fujikuroi* strains by estimating the conversion of ent-[3H] kaurene to ent-kaurenoids. They demonstrated the predominant product was ent-kaurenoic acid (> 90% of the total incorporation) in all incubations (Ashman et al., 1990), which indicated that the *Gibberella fujikuroi* kaurene oxidase was a particularly efficient enzyme. The genes for gibberellin biosynthesis pathway in *G. fujikuroi* are clustered on chromosome 4, including the bifunctional ent-copalyl diphosphate synthase/ent-kaurene synthase, a GA-specific geranylgeranyl diphosphate synthase, and four cytochrome P450 monooxygenases (Tudzynski et al., 2001). A knockout mutant of the P450-4 gene in *G. fujikuroi* resulted in the accumulation of ent-kaurene, while a GA-deficient mutant strain SG139, missing the entire 30-kb GA biosynthesis gene cluster, was able to convert ent-kaurene to ent-kaurenoic acid only after it had been transformed with copy of the P450-4 gene (Tudzynski et al., 2001, Malonek et al., 2005). These experiments thus confirmed the functional identity of kaurene oxidase genes from *Arabidopsis* and *Gibberella fujikuroi* kaurene oxidase, which could then be compared and evaluated for their utility for engineering kaurenoic acid production in yeast.

Grand Fir, *Abies grandis*, is a coniferous tree that produces resin acids rich in abietic acid. Abietic acid, like kaurenoic acid, is a diterpene acid derived from a hydrocarbon precursor, abietadiene. Abietadiene is synthesized by abietadiene synthase, ABS, a bifunctional enzyme which catalyzes two sequential cyclization reactions (Class I and Class II synthase reaction) in the formation of a mixture of abietadiene double bond isomers (Peters & Croteau, 2002a, Peters & Croteau, 2002b, Peters et al., 2000, Peters et al., 2001, Peters et al., 2003, Vogel et al., 1996). This reaction is analogous to that catalyzed by the *Gibberella fujikuroi* kaurene synthase of the gibberellin biosynthetic pathway. The first reaction converts GGPP to CPP via protonation-initiated cyclization, while the second reaction converts CPP to the abietadiene skeletons via ionization-
initiated cyclization (Peters & Croteau, 2002a, Peters & Croteau, 2002b, Peters et al., 2000, Peters et al., 2001, Peters et al., 2003). In A. grandis, abietadiene, like all diterpenes in plants, is biosynthesized in the plastid compartment, and thus abietadiene synthase contains a N-terminal plastidic targeting sequence, so-called targeting transit peptide, which is cleaved to form the mature protein when it is imported into the plastid compartment (Peters et al., 2000). Peters et al. generated a truncation series of the putative targeting sequence and evaluated “pseudomature” forms of the abietadiene synthase (Peters et al., 2000). Removal of 5’ DNA sequences encoding for the first 84 amino acid residues resulted in a bacterial expressed form of the enzyme that behaved as a functionally soluble enzyme, exhibited good catalytic activity, and produced the same chemical profiles of abietadiene, levopimaradiene, and neoabietadiene, as the native abietadiene synthase (Peters et al., 2000). We, hence, choose to determine if over-expression of the Grand Fir ABS gene with the sequence corresponding to the 84 N-terminal transit peptides deleted in yeast could result in abietadiene accumulation.

Like the conversion of kaurene to its acid form, the diterpene resin acids found in Grand Fir (Abies grandis) and lodgepole pine (Pinus contorta) are derived from GGPP being converted first to diterpene hydrocarbons like abietadiene, which then are subject to successive oxidation of the C18-methyl to yield the acid forms (Funk & Croteau, 1994, Funk et al., 1994). Funk and Crouteau (1994) also demonstrated that there were three different enzymatic activities catalyzing the sequential oxidation of abietadiene to abietic acid, with the first two steps being catalyzed by cytochrome P450-dependent monooxygenases, abietadine and abietadienol hydroxylases, while the third step, the conversion of abietadienal to abietic acid, is catalyzed by a soluble aldehyde dehydrogenase. Equally interesting, the two P450-dependent monooxygenases activities are inducible and reach maximum levels within 10 day after wounding, while the soluble aldehyde dehydrogenase activity seems to be constitutive (Funk et al., 1994). In fact, Ro et al. (2005) cloned and functionally identified a multi-functional and multi-substrate cytochrome P450 enzyme, CYP720B1, from Loblolly pine and classified this enzyme as abietadienol/abietadienal oxidase (PtAO) (Ro et al., 2005). When expressed in yeast, PtAO catalyzed a very low level of oxidation of abietadiene, but showed a clear preference for the oxidation of abietadienol or abietadienal (Ro et al., 2005, Ro & Bohlmann, 2006). Hamberger et al. (2011) have cloned two other sequence related P450 enzymes CYP720B4 from white spruce (Picea glauca) and Sitka spruce
(Picea sitchensis) (Hamberger et al., 2009, Hamberger et al., 2011). CYP720B4 (PsCYP720B4) was active with 24 different diterpene substrates, catalyzing consecutive C-18 oxidations in the biosynthesis of series diterpene alcohols, aldehydes, and acids. When co-expressed with a higher plant GGPP synthase, abietadiene synthase, and Cytochrome P450 reducatase (CPR), yeast were able to accumulate abietic acid up to 0.2 mg/L (Hamberger et al., 2011).
Figure 3.3. Biosynthetic conversion of geranylgeranyl diphosphate (GGPP) via copalyl diphosphate (CPP) to kaurene, then kaurenoic acid, or the conversion abietadiene then abietic acid.

The figure is also annotated to show how plants utilize the successive action of two enzyme for kaurene biosynthesis while fungi utilize a single multi-functional enzyme.
3.3. Results

3.3.1. Co-expression of a mutant prenyltransferase

Specific efforts were undertaken to generate yeast lines balanced for expressing high levels of heterologous proteins and in turn accumulating high levels of diterpenes. The first indication that the co-expression of a GGPP synthase might be needed along with a diterpene synthase was that neither the wild type yeast, nor our newly generated ZX yeast series with a sterol dispensable pathway and erg9 knock out accumulated any geranygeraniol (GGOH), which would be a sign that a GGPP pool exists in yeast. Another indication that GGPP was in short supply was our observation that only very low amounts of abietadiene accumulated in our ZX yeast line, which also over-expressed the ABS gene (Fig. 3.4). However, when expression of the ABS gene was coupled with the co-expression of a mutant avian farnesyl diphosphate synthase (mFPS F112A), those yeast lines demonstrated a dramatic improvement in their accumulation of abietadiene at the expense of farnesol accumulation (Fig. 3.5). The mutant FPP synthase, mFPS F112A, was previously characterized as exhibiting a preferential biosynthesis of GGPP, rather than FPP (Fernandez et al., 2000), and was additionally preferred because the encoded avian enzyme is relatively small, but particular active as a homodimeric protein, and because this foreign gene and protein would avoid any of feedback regulation that a native fungal GGPP synthase would be subject to.

The benefit of co-expressing the mutant FPS gene with other diterpene synthases corroborated the generality of this observation. In Figure 3.6, co-expression of the mFPS gene along with a codon optimized fungal kaurene synthase gene (Toyomasu et al., 2000) dramatically improved kaurene accumulation (lower panel) as observed for abietadiene biosynthesis (upper panel). Equally important to note, the enhanced diterpene accumulation due to the co-expression of the mFPS did not impose any obvious penalty in cell biomass accumulation (OD600 nm). Cell culture growth was, in fact, improved from 20 to 40% when the diterpene synthase genes were co-expressed with the mutant prenyltransferase.
Figure 3.4. Comparison of the terpene chemical profiles of yeast over-expressing the abietadiene synthase (ABS) gene versus control yeast not harboring the ABS gene.

GC chromatographs of extracts prepared from yeast engineered for expression of the abietadiene synthase (ABS) gene (upper panel) versus control yeast (those engineered with an empty plasmid DNA, no ABS gene) (lower panel). The yeast lines were grown for 5 days, aliquots of the culture were extracted into hexane, and the hexane extracts then profiled by GC-MS. Cedrene was added to cultures prior to extraction as an external standard to account for sample extraction efficiency (peak 1); farnesol (peak 3) was monitored as an estimation of how much carbon flux to FPP was occurring in the yeast cells; and abietadiene (peak 5) was monitored as a measure of how much terpene intermediates (IPP, DMAPP and FPP) were being diverted to diterpene biosynthesis.
Figure 3.5. Comparison of terpene chemical profiles from yeast co-expressing an alternative GGPP synthase (mFPS F112A) with abietadiene synthase versus only overexpressing the abietadiene synthase.

GC chromatograms of yeast co-expressing the abietadiene synthase (ABS) and a mutant avian farnesyl diphosphate synthase (mFPS) (upper panel) versus a yeast line only expressing the ABS gene (lower panel). The yeast lines were grown for 5 days, aliquots of the culture were extracted into hexane, and the hexane extracts then profiled by GC-MS. Cedrene was added to cultures prior to extraction as an external standard to account for sample extraction efficiency (peak 1); farnesol (peak 2) was monitored as an estimation of how much carbon flux to FPP was occurring in the yeast cells, thus estimating how much carbon was escaping channeling to diterpene biosynthesis; and abietadiene (peak 3) was monitored as a measure of how much isoprenoid intermediates (IPP, DMAPP and FPP) were being diverted to specialized diterpene biosynthesis.
Figure 3.6. The co-expression of the mFPS gene with different diterpene synthases genes enhances diterpene accumulation.

In the upper panel, yeast engineered for expression of the ABS gene or ABS gene plus mFPS gene were grown under standard conditions and aliquots of the cultures were monitored daily for growth (OD600 nm) and abietadiene accumulation (GC-MS determination). In the lower panel, yeast were engineered for expression of a second diterpene synthase gene, kaurene synthase, plus and minus co-expression of the mFPS gene. Cultures were monitored daily for growth (OD600 nm) and kaurene accumulation (GC-MS).
3.3.2. Identification of gene expression promoters and vector configurations to enhance kaurene diterpene accumulation

The successful accumulation of diterpenes in cultures when the mutant FPS and diterpene synthases were co-expressed suggested that the expression level of each gene relative to one another (the stoichiometric relationship) might be an important for optimized diterpene production. A variety of gene promoter combinations were then examined with the notion that this would be the best means for regulating the level of the target enzymes in the cells. The strategy for this effort is outlined in Fig. 3.7. Varied gene promoter elements driving expression of the mutant FPS gene on one plasmid construct in combination with the same promoters directing expression of the fungal kaurene synthase (FKS) gene on an another plasmid construct were examined in yeast for kaurene production. The promoter elements included the actin (ACT1) promoter (Mateus & Avery, 2000), a copper inducible (CUP1) promoter (Tohoyama et al., 2001), glyceraldehyde phosphate dehydrogenase (GPD) promoter (Bitter & Egan, 1984), transcription elongation factor (TEF1) promoter (Mumberg et al., 1995), and the alcohol dehydrogenase (ADH1) promoter, which we previous described using for heterologous expression in yeast (Takahashi et al., 2007). ADH, TEF and GPD promoters are strong constitutive promoters and their relative strength arranged in ascending order, with ADH1 being the moderate strength, and the GPD promoter the strongest. The Act1 promoter is a relatively lower constitutive promoter, and appeared as the strongest in cells growing actively with sucrose (Monfort et al., 1999). While the CUP1 promoter belongs to the class of promoters known for their inducibility, exhibits a rapid induction of approximately 20-fold and the maximal level of transcript detected 30 min after exposure to copper (Monfort et al., 1999, Romanos et al., 1992). These various strength and inducible promoters combination of co-expression of prenyltransferase and diterpene synthase helps to identify the best fine tuning association between these two enzymes for carbon flux to build the desired diterpene compound.

Yeast strain ZX 2-2 was co-transformed with the various two plasmid construct combinations, then individual transformant lines were monitored for kaurene and farnesol accumulation (Fig. 3.8). While we were obviously screened these lines for the promoter combination giving the highest level of diterpene production, an equal important parameter was the farnesol levels. If a yeast line was efficiently diverting the earlier isoprenoid precursors to diterpene, their farnesol levels would be expected to be
equally low. By these criteria, having the GPD promoter direct expression of both the mutant prenyltransferase and the kaurene synthase genes yielded the highest level of kaurene with the greatest efficiency. The results of Figure 3.8 also demonstrate that the absolute level of gene expression and stoichiometry of the encoded enzymes can significantly influence overall diterpene production.

We further assessed this by assembling the prenyltransferase and diterpene synthase genes into a single vector. In this way, we were evaluating whether variation in diterpene accumulation could be associated with possible variation in gene copy number as reflected by possible variation in plasmid copy number caused by different auxotrophic selection markers on vectors, or whether a one-to-one stoichiometry of prenyltransferase and diterpene synthase genes on a single plasmid vector were preferable. In the first examination of these possibilities, the constructs were substituted GPD, ACT, TEF, and ADH promoters to drive expression of the prenyltransferase and diterpene synthase genes in single vector. The constructs were then introduced into yeast and multiple, independent transformants selected for monitoring diterpene (kaurene) production and farnesol accumulation (Fig. 3.9). Farnesol accumulation was monitored as a measure of how much carbon was not efficiently being converted to diterpene. Once again, the combination of the dual combination of the GPD promoters proved superior to any other promoter combination with respect to kaurene yield and efficiency, as noted by the limited amount of farnesol accumulating. But no significant improvement of production was noted in comparison with two genes on separated vectors. At this point, the most optimal vector design suggested by the experimental findings recommends that having both the prenyltransferase and diterpene synthase genes driven by the GPD promoter was the preferred structural organization.
Figure 3.7. Construct design for testing the importance of specific gene promoters for diterpene (kaurene) production in yeast.

A variety of promoter elements were inserted independently upfront of the mFPS gene and the fungal kaurene synthase gene, then yeast were transformed with all possible combinations of each construct. The different transgenic yeast lines were then evaluated for kaurene production levels. Pact = the actin gene promoter (Mateus & Avery, 2000); Pcup = the copper inducible promoter (Tohoyama et al., 2001); Pgpd = the glyceraldehyde phosphate dehydrogenase promoter (Bitter & Egan, 1984); Ptef = the transcription elongation factor (tef1) promoter (Mumberg et al., 1995); Padh = the alcohol dehydrogenase promoter (Takahashi et al., 2007).
Figure 3.8. Kaurene and farnesol (FOH) accumulation in yeast engineered for expression of the kaurene synthase and mutant avian FPP synthase driven by different gene promoters.

Yeast strain ZX 2-2 were transformed with the various plasmids noted in Fig. 3.7, selected for prototrophic growth without leucine or histidine added to the culture media (standard recipe), then grown for 10 days before extracting and chemically profiling aliquots of the cultures by GC-MS. For the line harboring the CUP promoter construct, the cultures were grown for 2 days, then 1 mM copper sulfate was added to the growth media.
Figure 3.9. Kaurene, farnesol (FOH), and geranylgeraniol accumulation in yeast engineered for expression of the kaurene synthase and mutant avian FPP synthase driven by different gene promoters combinations on a single expression vector.

Evaluating the efficiency of he ACT, ADH, TEF or GPD promoters to direct expression of both the mutant prenyltransferase and kaurene synthase genes on a single plasmid vector for the diterpene kaurene production. Yeast line ZX 2-2 was transformed with the indicated plasmid vector (i.e. 2act = the actin promoter driving expression of both the kaurene synthase gene and the mutant FPP synthase gene arrayed on a single expression vector harbor the Leu2 auxotrophic selection marker) and a resulting transformant line chemically profiled by GC-MS after 10 days of growth in standard section media.
3.3.3. Optimization of culture conditions to enhance kaurene diterpene accumulation

Throughout our testing of genes and genetic elements for enhancing diterpene accumulation, we noted some variation in diterpene yields with the culture conditions. The yield of diterpene compounds depend primarily on maximizing and balancing expression levels of the heterologous proteins, and the cell concentration which is directly related to biomass accumulation. Thus the medium composition and culture condition can affect cell growth and protein yield and thereby affect the target compound yield. We therefore examined these parameters more systematically and found that for each diterpene target, specific culture conditions could dramatically influence overall diterpene accumulation. Complex complete medium usually supports higher cell growth rates in comparison with minimal media because it provides an excess of essential nutrients and provides an exogenous source of some factors which the cells do not need to synthesize. There are also two kinds of selection markers used in the molecular manipulations of yeast. Antibiotic resistant genes provide for selection against antibiotic addition to the cultures, and auxotrophic gene markers provide for a trans-complementation for knockout mutation in key enzymes of primary metabolism. The relatively high price for antibiotic compounds precludes their use in large-scale fermentations, hence auxotrophic markers are the preferred selection markers used for molecular manipulations in yeast. However, when using yeast strains harboring numerous auxotrophic mutations, only some of which are complemented by introduced gene constructs, the concentrations of essential nutrients to be added to the growth medium for optimal culture growth and heterologous expression of engineered genes must be empirically determined. Pronk et al. (2002) demonstrated that the auxotrophic amino acid concentrations are limited in many standard synthetic medium recipes for cultivation of *S. cerevisiae* (Pronk, 2002). In such cases, yeast growth is limited by the concentration of the auxotrophically required nutrient in the medium. Pronk recommended much higher concentrations of commonly applied auxotrophy complementing compounds than the standard recipes based on calculations of the required compound content in 0.5 g biomass per gram glucose during aerobic growth condition (Pronk, 2002). We presumed doubling the auxotrophical amino acids concentrations recommended by Pronk would avoid nutrient limitation conditions. To test the yeast performance in different nutrients media, yeast ZX 2-2 strain were cultured in
three kinds of media, standard selection media, YPDE rich media and the optimized selection media. Yeast culture samples were collected after 6 days and 12 days of growth and evaluated for the amount of farnesol they had accumulated. Both rich media and optimized media dramatically enhance farnesol level two folds compared with the standard selection media (Fig. 3.10 A). To further corroborate these observations, a yeast line expressing both the mutant FPP synthase and kaurene synthase genes (both driven by the GPD promoters, on two separate vectors were inoculated into YPDE nutrient rich media and the optimized selection media in shake flask. As shown in Fig. 3.10 B, cell growth and terpene accumulation were determined by spectrophotometer or GC-MS every two days. The kaurene producing yeast line grew better in the rich complete media than in optimized selection media with 150% higher OD$_{600}$ reading. Kaurene accumulation level was also slightly higher in rich nutrient media than optimized selection media. The more interesting result was that the farnesol levels were extremely low, less than 2 mg/L, and no significant level of geranygeraniol was detected under these growth conditions, which suggested that sufficient conversion of FPP to GGPP and then to diterpene hydrocarbon had occurred. When the engineered yeast were cultivated in a 7.5 L bioreactor, 569 mg/L kaurene accumulated with only 41 mg/L of GGOH and 25.8 mg/L of FOH accumulating (Fig. 3.11).
Figure 3.10. Optimizing medium conditions to enhance terpene production by yeast strain ZX 2-2

A) Yeast ZX 2-2 line was cultured in three medium for the indicated time periods indicated, and the amount of farnesol accumulating in these cultures determined by GC-MS. B) Yeast 2-2 transformed with the plasmids containing the kaurene synthase and prenyltransferase genes under GPD promoter on two separate expression vector plasmids were cultured under optimized selection media or complete media, and monitored for growth (OD 600) and terpene levels by GC-MS.
Yeast 2–2 transformed with plasmids containing the kaurene synthase and prenyltransferase genes under the control of the GPD promoters on two separate vectors were cultured in complete rich media at 30°C in a 7.5 L stirred-tank bioreactor. Culture growth was measured at OD$_{600}$ nm and terpene accumulation was determined by GC-MS.
3.3.4. Screening yeast strains for optimal accumulation of a second diterpene, abietadiene

To determine if we could extend our findings with kaurene biosynthesis and accumulation to a second diterpene, we mimicked many of the engineering and culture conditions for abietadiene biosynthesis. Yeast ZX 2-2 strain was transformed with constructs for the prenyltransferase and abietadiene synthase genes on one plasmid vector, or on separate vectors with all genes driven by GPD promoter, then cultivated in optimized selection media monitoring growth and diterpene accumulation. Surprisingly, those transgenic lines with the multiple plasmid constructs exhibited relatively minor variation in the level of diterpene and farnesol accumulated, while the lines transformed with the single vector harboring both the prenyltransferase and diterpene synthase genes showed more than 50% variation in the absolute levels of farnesol and abietadiene (Fig. 3.12). Nonetheless, independent transgenic lines containing the targeted genes on a single plasmid vector also demonstrated greater than 30% more abietadiene and farnesol than when the transgenes were introduced on separate plasmid vectors. When combined with the information for kaurene production in Figs. 3.8 and 3.9, the most optimal vector design suggested that having both the prenyltransferase and diterpene synthase genes on one plasmid vector and having expression of both genes driven by the GPD promoter was the preferred structural organization. However, the abietadiene accumulation in yeast was still relatively low relative that observed for kaurene production earlier, and the large amount of FOH accumulated in these lines would suggest that the conversion of GGPP to abietadiene was limiting. That may be caused by the Garnd Fir abietadiene synthase specific activity being relatively low in comparison to the fungal kaurene synthase, or it might arise from some sort of feedback regulatory mechanism by GGPP.

Ten additional ZX yeast lines plus ZX 2-2 expressing the mFPS and abietadine synthase under the direction of the GPD promoter on a single plasmid construct were thus quantified for GGHO, abietadiene, and FOH accumulation. Three independent transformants of each strain were cultivated in test tube scale at 23°C. Although there is some variability between independent transgenic lines, reduction of the culture temperature from 30°C to 23°C significantly enhanced the abietadiene yield and efficiency as noted by the limited about of farnesol and geranylgeraniol accumulating (Fig. 3.13). Because line #31 of strain ZX178-08 accumulated the highest levels
abietadiene and lowest levels of FOH and GGOH, this line was chosen for further characterization and optimization for abietadiene production.

In Fig. 3.14, the accumulation of abietadiene, farnesol and geranylgeraniol by the ZX178-08 line over-expressing the mutant prenyltransferase and abietadiene synthase genes grown under 4 conditions (2 temperatures and 2 media) was examined. Not unexpectedly, when grown in nutrient rich media (YPDE), the yeast grew approximately 2-fold greater than when cultured in selection media (SCE) for 10 days. However, growth of the cultures at 23°C versus 30°C had relatively little influence over this 10-day period in terms of overall biomass accumulation. However, a dramatic effect on abietadiene accumulation was noted when the cultures were grown at 23°C in selection media. Abietadiene accumulation was 2-fold greater under these conditions than when grown at the higher temperature or in the nutrient rich media. The latter observation might be explained by the loss of the recombinant expression plasmid from the yeast grown in the absence of selection pressure provided by the selection media. In contrast, kaurene accumulation by yeast co-expressing the fungal kaurene synthase and mutant prenyltransferase also under the control of GPD promoters was highest in yeast grown in nutrient rich media rather than selection media, even if the cultures were grown for 10 or more days (see Figs. 3.10 and 3.11). But cooler culture temperatures appear to improve diterpene accumulation regardless of the diterpene synthase gene used.

There are two major types of plasmid instability associated with yeast as we have prepared and grown segregation and structural instability. Segregation instability is due to the failure to distribute plasmids evenly between daughter cells during cell division resulting in reduction of copy number and loss of plasmid. Structure instability is due to the mutation or changes in the structure of the plasmid such that rearrangements or loss of plasmid DNA sequences through recombination might occur. Plasmid instability in the yeast strains might also be caused by the toxicity of the high level constitutive expression of ABS gene and accumulation abietadiene products resulting in poor cell growth, and plasmid segregation instability. The repeat DNA sequences of GPD promoter and ADH terminator on the single plasmid, might also improve the probability of homologous recombination resulting in gene sequence loss. Nonetheless, the experimental evidence up to this point for the most optimal vector design suggested that having both the prenyltransferase and diterpene synthase genes on one plasmid vector
and having expression of both genes driven by the GPD promoter was the preferred structural organization.

When the ZX178-08 yeast were grown in a 7.5 L bioreactor, 207 mg/L abietadiene, 20 mg/L palustradiene, 58 mg/L levopimaradiene, 58 mg/L neoabietadiene accumulated with only 0.8mg/L and 3.6 mg/L GGOH and FOH, respectively (Fig 3.15). These additional diterpene products were identified by careful GC analysis and identification by comparison of retention time and mass spectrum to those of authentic standards as well as reference to literature documentation (Lee et al., 2001). These abietadiene isomers were previously identified in in vitro assay using recombinant ABS protein or ABS protein extracted from Grand Fir as described by Peters et al. (2000).
To test for how the molecular configuration of the mutant prenyltransferase (mFPS, geranylgeranyl diphosphate synthase) relative to the diterpene synthase (ABS, abietadiene synthase) might influence diterpene production, the indicated plasmid vectors were introduced into yeast ZX 2-2 and 4 independent transgenic lines grown for 10 days at 30°C in 30 ml optimized selection media prior to extracting and chemically profiling by GC-MS for abietadiene and farnesol (FOH) accumulation.

**Figure 3.12.** Comparison of the vector configuration effects on abietadine production in ZX 2-2 yeast line.
Figure 3.13. Quantitation of abietadiene production levels in the ZX yeast series lines.

The indicated yeast lines were engineered with both the mutant prenyltransferase and abietadiene synthase genes, and 3 independent transformants for each strain were grown as test tube shake cultures at 30°C with 3 ml of optimized selection medium containing 40 µg/ml of ergosterol for 6 days prior to sampling the cultures. One ml aliquots of cultures were extracted with n-hexane, then subjected to GC-MS. Abietadiene, GGOH, and FOH levels were quantified relative to an external standard. * indicates lines selected for further evaluations.
Figure 3.14. Culture medium and temperature influences on abietadiene accumulation.

Yeast strain ZX178-08 co-expressing abietadiene synthase and mutant prenyltransferase under the control of the GPD promoter on one vector was grown in nutrient rich media (YPDE) or optimized selection media (SCE) at 23°C or 30°C for 10 days. Culture growth was measured at OD$_{600}$ nm and terpene accumulation was determined by GC-MS.
Terpene chemical profiles of yeast strain ZX178-08 co-expressing abietadiene synthase and mutant prenyltransferase grown under fermentation conditions.

Yeast ZX178-08 transformed with a single plasmid containing the abietadiene synthase and prenyltransferase genes under the control of the GPD promoters was cultured in optimized selection media at 23°C in a 7.5 L stirred-tank bioreactor. Culture growth was measured at OD$_{600}$ nm and terpene accumulation profiles were determined by GC-MS.
Figure 3.16. GC-MS assessment of the diterpene hydrocarbons accumulating in yeast co-expressing the genes encoding for abietadiene synthase and a mutant prenyltransferase.

When co-expressing the mFPS F112A gene and the abietadiene synthase gene under the regulation of the GPD promoter in ZX1708-08 lines, several abietadiene isomers accumulate, as shown in the GC chromatogram of an extract prepared from a culture grown for X days in selection medium at 23°C as a shake flask culture (A). The identities of the compounds in the various peaks were determined by matches of their mass spectra data to diterpenes reported in the NIST library (B).
3.3.5. Decorating diterpene hydrocarbon scaffolds

Having achieved the production of diterpene hydrocarbon production in yeast, the next objective sought the biosynthesis of more highly modified forms of diterpenes and especially those molecules that might have industrial, agricultural or medicinal applications. For this purpose, we have utilized a 3 plasmid construct design (Fig. 3.17). Plasmids 1 and 2 are those described above and whose expression in yeast yields robust levels of diterpenes such as kaurene. The third plasmid construct was similarly designed to constructs 1 and 2, but contained a gene encoding for kaurene oxidase, a fungal P450 enzyme (Tudzynski et al., 2001) or Arabidopsis kaurene synthase, both which require reducing equivalents from a cytochrome P450 reductase (CPR) (Takahashi et al., 2007) for activity as shown in Fig. 3.17A. Three vector constructs coding mfps, FKS, along with GFKO plus CPR or AtKAO plus CPR were transformed into yeast line ZX2-2 and confirmed transformants evaluated for their efficiency of converting kaurene to its oxidized form, kaurenoic acid. Fig 3.17 B shows GC chromatograms of yeast culture extracts prepared from transgenic yeast and derivatized to assure volatility for detection by gas chromatography. Apparently, the over-expression of the fungal kaurene oxidase gene results in a more efficient oxidization of the C18 methyl on kaurene scaffold. When transgenic yeast from test tube culture were quantified, the kaurene scaffold chemicals composition percentage distribution were 61.4% kaurene, 28.5%, kaurenal, 5% kaurenol, 5.3% kaurenoic acid from AtKAO transgenic yeast lines, versus 21% kaurene, 13.1% kaurenal, 6% kaurenol, 53% kaurenoic acid from the GFKO transgenic yeast line. Hence, over-expression of the fungal kaurene oxidase gene appears to result in more enzyme activity that catalyzes the successive three-step oxidation of kaurene.

This obtained kaurenoic acid producing line was evaluated for diterpene production at 23°C and 30°C in nutrient rich media and optimized selection media as described previously for simply hydrocarbon production (compare Fig. 3.19 to Fig. 3.14). The yeast were grown for 10 days before the accumulation of kaurene and its specific oxidation products kaurenal and kaurenoic acid were measured by GC-MS. Consistent with the earlier observations for only kaurene production, maximal production of approximately 200 mg/l of kaurenoic acid was observed for the culture grown in nutrient rich media at the reduced temperature. The diterpene productivity was about 2-fold greater than the any of the other conditions tested. These growth conditions were corroborated when this
A yeast line was cultivated in a 7.5 L bioreactor. Under these conditions, 514 mg/L kaurenoic acid accumulated with levels of kaurene and kaurenal reaching 71.7 mg/L and 20.1 mg/L, respectively (Fig. 3.20).

Because the kaurenoic acid accumulating yeast requires an exogenous source of sterol for growth, as do all the third generation ZX strains, we next examined the optimal sterol source and concentration on the overall diterpene production of the yeast. Ergosterol and cholesterol were tested for their effects on kaurenoic acid and other terpene production at different concentrations and at 2 stages of culture development in shake flask cultures (Fig. 3.21). Too low or too high a concentration of ergosterol or cholesterol suppressed diterpene production, yet total amount of kaurene and its oxidized products and specifically kaurenoic acid production reached 660 mg/L and 470 mg/L, respectively in medium containing 10 mg/L ergosterol in shake flask culture. Additional improvement in diterpene productivity might be possible if the sterol requirement were met with a fed-batch application. Bulk addition of the sterol at the start of the culture necessitates its addition with large amounts of detergent, which could have adverse effects on overall culture physiology and productivity, and a more regulated and titratable addition of the required sterol might alleviate some of these effects.

Efforts to generate yeast lines accumulating oxidized forms of abietadiene were less successful. Two cytochrome P450 genes noted in the literature for their ability to code for enzymes hydroxylating abietadiene are CYP720B1 from Loblolly pine described as an abietadienol/abietadienal oxidase (PtAO) (Ro et al., 2005) and that from white spruce, CYP720B4, (Hamberger et al., 2009, Hamberger et al., 2011) were evaluated. When PtAO was co-expressed in abietadiene accumulating yeast, no oxidized products were observed, nor when co-expressed along with GFKO assuming that this enzyme might initiate the first oxidation at C18 to yield abietadienol (data not shown). When the white spruce CYP720B4 gene was co-expressed in abietadiene producing line, approximately 5 mg/L abietic acid accumulated. Whether this inefficient conversion could be due to low expression levels of the corresponding P450 genes, inadequate balancing of the various enzymes involved, or simply low catalytic efficiency of the cytochrome P450 enzymes themselves remains to be determined.
Figure 3.17. Test for the specificity and efficiency of kaurene oxidation by the co-expression of an Arabidopsis kaurene oxidase gene or the Gibberella fujikuroi kaurene oxidase gene in yeast optimized for kaurene production.

In panel A, the indicated plasmid vectors containing a P450 reductase gene (CPR) along with either the Arabidopsis kaurene oxidase gene under the transcriptional control of a cup promoters, or a fungal kaurene oxidase gene under transcriptional control of the GPD promoter were transformed into the highest kaurene accumulating lines of yeast ZX 2-2. In panel B, the chemical profiles of the respective yeast lines co-expressing the mutant prenyltransferase gene, the kaurene synthase gene and either of the kaurene oxidase genes shown in panel A were determined by GC-MS. The mass spectra data for the kaurenoic acid and other oxidized species are also shown.
The mutant prenyltransferase (mFPS) and kaurene synthase (FKS) constructs were described above and the new construct consists of a gene encoding for a fungal P450 enzyme catalyzing the oxidation of kaurene to its acidic form (kaurene oxidase, GFKO), plus a cytochrome P450 reductase (CPR) that provides reducing equivalents to the kaurene oxidase. Expression of these genes, like the prenyltransferase and kaurene synthase genes, is controlled by the GPD promoter.

**Figure 3.18.** Expression constructs designed for producing diterpene acids in yeast.

GFKO: Fungal Kaurene oxidase
CPR: Cytochrome P450 reductase
Figure 3.19. Culture medium and temperature influence kaurenoic acid accumulation.

In panel A, yeast strain ZX178-08 co-expressing the mutant prenyltransferase, kaurene synthase, CPR and kaurene oxidase genes under the direction of the GPD promoters as illustrated in Fig. 1.8, were grown in nutrient rich media (YPDE) or optimized selection media (SCE) at 23°C or 30°C for 10 days. Culture growth was then measured at OD600 nm and kaurene, kaurenal, kaurenoic acid accumulation was determined by GC-MS after derivatization treatment. In panel B, the distribution of the various diterpenes between the culture medium and the cell pellet are presented.
Terpene chemical profiles of yeast strain ZX178-08 engineered for kaurenoic acid biosynthesis as shown in Fig. 3.18 grown under fermentation conditions.

Yeast strain ZX178-08 harboring all the vectors shown in Fig. 3.18 was grown in nutrient rich media at 23°C in a 7.5 L stirred-tank bioreactor. At the indicated time points, culture growth (OD$_{600}$ nm) and terpene accumulation (determined by GC-MS) were measured.
Figure 3.21. Sterol dependence of engineered yeast cultures to accumulate kaurene, kaurenal and kaurenoic acid.

Yeast strain ZX178-08 harboring all the vectors shown in Fig. 3.18 was grown in 30 ml nutrient rich medium at 23°C in shake flasks containing the indicated concentrations of cholesterol or ergosterol. At the indicated time points, culture growth was measured as OD$_{600}$ nm and terpene accumulation determined by GC-MS.
3.4. Discussion

Impressive progress in diterpene engineering in microbes has been achieved over the last decade or so. Taxadiene production started at 1.3 mg/L in *E. coli* (Huang et al., 2001) and 1 mg/L in yeast (Dejong et al., 2006), and recent advances reported 1 g/L in *E. coli* (Ajikumar et al., 2010) under fed-batch fermentation with a multivariate-modular approach for optimizing, balancing and channeling the overflow flux from IPP and DMAPP toward diterpene biosynthesis. Tokuhiro et al. (2009) overexpressed fusion enzymes to increase the association of successive acting enzymes and channel of intermediates to desired products, yielding about 238.8 mg/L geranylgeraniol in yeast shake flask cultures. Using similar strategies, miltiradiene, the diterpene precursor to the Chinese herb medicine tanshionone, was produced up to ~480 mg/L in optimized yeast fermentation (Zhou et al., 2012, Dai et al., 2012).

These developments have built on important observations that have been reported to influence terpene metabolism in yeast. First, it has been recognized for quite some time that carbon flux through the mevalonate pathway is highly regulated. HMGR, for instance, is considered to be a rate-limiting step in this pathway (Gardner and Hampton 2009, Liscum et al., 1985). Over-expression of truncated, heterologous forms of this enzyme along with changes in the transcriptional promoter driving expression of this engineered gene to overcome native regulatory mechanisms can dramatically increase the amount of squalene accumulation (Dai et al., 2012, Polakowski et al., 1998). An up-regulation mutation in the general transcription factor gene *upc2* also increased the uptake of exogenous sterol from the medium five fold, paving the way for using this mutation to creating sterol auxotrophic mutants in yeast (Crowley et al., 1998). Over-expression of other genes within the mevalonate pathway (*ERG 13, 12, 8, 1, 11, 24, 25, 6, 2, 3 and IDI*) also dramatically improved sesquiterpene production in yeast harboring the *upc2* mutation and truncated HMGR gene cassette (Westfall et al., 2012).

In the current work, a novel yeast line was developed with the intent that this strain could be utilized for the production of all classes of terpene compounds. The aim of the work reported in this chapter was to optimize these yeast strains for diterpene production and more specific for kaurene, kaurenioic acid and abietadiene. The strategy employed was first to assume that the substrate, GGPP, for diterpene biosynthesis would be limiting in the yeast, and this necessitated the incorporation of a GGPP synthase into our yeast strains in order to divert the available IPP, DMAPP and FPP for GGPP biosynthesis.
While others have utilized functionally characterized GGPP synthase genes to effect such a change in metabolism (Dai et al., 2012, Tokuhiro et al., 2009, Zhou et al., 2012), we instead chose to mutate the avian FPP synthase into a GGPP synthase for this purpose. Our reasoning was that the avian FPP synthase is a superior prenyltransferase exhibiting higher catalytic turnover rates and lower Kms for its substrates than other prenyltransferase (Tarshis et al., 1996), and because the extensive biochemical and structural characterization of this enzyme readily facilitated its conversion into a potent GGPP synthase (Tarshis et al., 1996). We also assumed that the conversion of GGPP to diterpenes would be subject to the same enzymological concerns. That is, higher plant diterpene synthases are known to have low catalytic efficiencies (Keeling et al., 2010). Hence, we screened both plant and fungal sources for appropriate diterpene synthases. The same screening effort was directed to the enzymes that can decorate the diterpene scaffolds, and in particular the diterpene hydroxylases. This was followed by screening for the best expression vectors for the transgenes introduced into our yeast strains and lead to the identification of the GPD promoter as the best promoter leading to the highest levels of the desired diterpenes. Further enhancement of diterpene production by our yeast strains was provided by documentation of specific culture medium and temperature conditions, which varied depending on the desired diterpene product.

We fully recognize that additional efforts can improve the diterpene yield in our yeast lines. For example, recent precedent for improving chemical yields by focusing on the association between enzymes catalyzing sequential reactions is an avenue worth considering. Dueber et al. (2009) built synthetic protein scaffolds bearing interaction domains allowing the assembly of heterologous proteins in prescribed stoichiometries for three mevalonate biosynthetic enzymes and achieved a 77-fold improvement in terpene product accumulation. Leonard et al. (2010) demonstrated that they were able to use a combinatorial mutation library of geranylgeranyl diphosphate synthase and levopimaradiene synthase to assemble enzyme complexes yielding increased production of levopimaradiene. Wild type levopimaradiene synthase, like many other terpene synthase catalyzes the production of several other diterpene products in addition to levopimaradiene. The key mutants selected by Leonard et al. (2010) not only increased diterpenoid production, but also tuned the overall product selectivity towards levopimaradiene. These two studies offer additional approaches for how we might further enhance kaurene and abietadiene diterpene production in our yeast platform.
A second research area warranting further investigation based on our results relates to plasmid stability and copy number. We observed that the greatest yield of kaurene and its oxidation products was in fermentation conditions using a nutrient rich cultivation medium. This is in contrast to cultures for abietadiene production, which were grown in selection medium, medium that uses auxotrophic selection markers for the maintenance of the engineered plasmid vectors. We do not know why in one case (abietadiene production) selection pressure for plasmid maintenance is required, but not in the other (kaurene production). However, the level of plasmid retention in a culture is known to be affected by both genetic and physiological factors. Plasmid instability has been divided in two cases, segregation instability and structure instability, and both cause loss of recombinant genes in host cells. When yeast cells are grown under non-selective conditions, even the most stable 2µ-based chimeric plasmids undergo significant segregational instability (Romanos et al., 1992, Mendoza-Vega et al., 1994). Leu2d and Ura3d are two frequently used defective variant of LEU2 and URA3 genes, having mutations within their promoter regions that dampen the expression level of the corresponding selection marker genes. Selection of yeast transformants harboring these mutant selection markers can result in very high copy numbers of the respective plasmids, such as plasmids containing the Leu2d mutant results in 200 copies per haploid genome, which is 5 to 10 higher than for the conventional LEU2 marker (Mendoza-Vega et al., 1994). However, Ro et al. (2008) observed that using the Leu2d selection marker for introducing the amorphadiene (a sesquiterpene) biosynthetic gene only increased production 1.3-fold in selection medium, yet production was 5.9-fold in to non-selective, rich medium. While these defective auxotrophic selection markers increase plasmid copy numbers, it remains to be determined if this amplification actually translates to increase gene expression of genes carried on the respective plasmid vector. Our screen here of the various promoters driving expression of the diterpene biosynthetic genes would suggest that a similar screen with the defective selection marker vectors may be helpful in this regard.

The third area of potential further optimization is in the culture medium and growth conditions. While we observed distinct differences in diterpene production based on nutrient rich versus selection medium, growth at 23°C versus 30°C, and exogenous sterol type and concentration, we are confident that many other growth condition parameters remain to be optimized. The efficiency of biomass accumulation by yeast is
dependent on the cells glucose consumption rate, which supports aerobic respiration and biomass accumulation, while anaerobic respiration encourages ethanol accumulation that inhibits cell growth. Thus, precise control of glucose addition to fermenting yeast can have a dramatic effect on secondary metabolism and the cultures abilities to accumulate diterpenes. In fact, it is more complicated than this simple model might suggest. Westfall et al. (2012) demonstrated using an ethanol and glucose pulse feeding strategy they could significantly boost amorphadiene production in fed-bath cultures. Since our yeast line is dependent upon an exogenous sterol supply, which is readily taken up under aerobic conditions, a multi-factorial screen of nutrients supplied by cultures grown under standard fermentation conditions (dissolved oxygen levels, pH control and impeller rpm) has the potential to also improve diterpene production levels.

The yeast strains used for our studies are haploid and there is evidence to suggest their conversion to the diploid state could also improve diterpene yield. Prototrophic diploid cells generally exhibit more tolerance to environmental stresses, for instance, osmotic pressure, generally exhibit good growth kinetics, and accumulate more biomass in a low cost medium. Zhou et al. (2012) recently demonstrated that converting a haploid yeast strain to their corresponding prototrophic diploid form improved the accumulation of the diterpene miltiradiene 2.5 folds. Efforts to convert the ZX series yeast to their diploid form are a clear priority objective for the future.

3.5. Materials and Methods

3.5.1. 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. YPD media for growing yeast without selection consisted of 1% Bactoyeast extract, 2% Bacto-peptone, and 2% glucose (or 0.5% glucose for select experiments). YPDE media was YPD media supplemented with ergosterol (40 mg/L) for ergosterol dependent lines.

Standard minimal selection media, SCE (pH 5.3), contained 0.67% Bacto-yeast nitrogen base (without amino acids), 2% dextrose, 0.6% succinic acid, 0.14% Sigma yeast
dropout solution (-his,-leu,-ura,-trp), uracil (20 mg/L), L-tryptophan (20 mg/L), L-histidine (20 mg/L), L-leucine (20 mg/L) and 40 mg/L ergosterol. The optimized selection meida is similar as the standard selection media but the supplement amino acid concentration used to complement auxotrophic genes are higher at uracil (300 mg/L), L-tryptophan (150 mg/L), L-histidine (250 mg/L), L-methionine (200 mg/L), L-leucine (1 g/L). Cholesterol and ergosterol stocks were 10 mg/mL in 50% Triton X-100, 50% ethanol, consideration sterilized and kept at -20°C. Selection media was prepared similarly except without supplementing the media with the indicated reagent based on the yeast auxotrophic makers. All solid media plates were prepared with 2% Bacto-Agar.

3.5.2. Yeast strains

The ZX yeast lines used in these studies were described in Chapter 2. Essentially, these strains were selected for their ability to utilize exogenous sterol sources under aerobic conditions and were engineered with a knockout mutation in their squalene synthase (ERG9) gene such that the basic mevalonate biosynthetic pathway is operative up to FPP biosynthesis.

3.5.3. Yeast transformation and culture performance

Yeast strains were transformed with the respective vector constructs using the FROZEN-EZ Yeast Transformation II Kit (Zymo Research, Irvine, CA) according to the manufacturer’s recommendations. About 1 µg of plasmid DNA was used per transformation, followed by selection on agar plates of SCE medium lacking specified amino acids for the auxotrophic markers (selection media) or YPDE (rich media) at 30°C. Variable numbers of independent colonies were subsequently picked and used to start 3 mL cultures in minimal media to characterize their terpene production capacities. Aliquots of these cultures were analyzed for terpene production after 3-6 days of incubation at 30°C with shaking by GC-MS. 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 30 mL shake flask cultures. Starter cultures grown to saturation in minimal media were inoculated into 30 mL SCE or YPDE media and 1 mL aliquots withdrawn at indicated intervals for up to 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 by GC-MS similar to the initial screening method.
3.5.4. GC-MS detection and quantification of terpenes

To determine terpene accumulation levels, aliquots of cultures grown for 3 to 12 days were extracted with hexane and aliquots evaluated by GC-MS. In general, to 1 volume of culture, 1 volume of acetone was added and mixed vigorously for 3 to 5 min to lyse the cells. The sample was then allowed to incubate at room temperature for 10 min before adding 1 volume of hexane containing a known amount of cedrene external standard. The mixture was again mixed vigorously, then centrifuged in a clinical centrifuge for 5 min at maximum speed. The upper organic layer was collected and when necessary, concentrated under a N₂ stream to 1/10 the original volume. An aliquot of the organic phase (1 μL) was then analyzed by GC-MS with a Varian CP-3800 GC coupled to a Varian Saturn 2200 MS/MS (Varian Medical Systems) using a Supelco SLB-5ms fused silica capillary column (30 m x 0.25 mm x 0.25 μm film thickness, Supelco). The initial oven temperature was set at 70 °C for 1 min, ramped to 200 °C at 8 °C/min, and then ramped to 300 °C at 20°C/in and held for 5 min more. Farnesol and diterpene levels were calculated relative to the cedrene external standard.

To determine kaurenoic acid accumulation level, yeast sample were collected and treated with acetone but extracted with 80% n-hexane and 20% ethyl acetate spiked with 4-octyl benzoic acid (20 µg/mL). The extracts were completely dried under a N₂ stream, resuspended in 100 μL methanol, and then derivatized with 60 μL of trimethylsilyl-diazomethane (Aldrich 527254) for 20 minutes at room temperature. Sample were completely dried under N₂ stream and resuspended in 100 μL hexane, 1 μL sample was subjected to quantitative and qualitative analysis by GC–MS.

3.5.5. Expression vector construction

The yeast GPD promoter (Pgpd) was amplified from the PYM-N14 plasmid described by Janke et al. (2004) using the primers GPD-BamHIF and GPD-NotIR primers and inserted into the pESC-His vector digested with BamHI and NotI to replace the original GAL1/10 promoters. The resulting plasmid was named pESC-His-gpd. The other promoter elements were obtained similarly. Act promoter was amplified from the yeast genomic DNA described by Mateus et al. (2000). ADH1 promoter was amplified from Yep352 plasmid (Takahashi et al., 2007). Tef1, Cup1 promoters were amplified from the plasmids PYM-N18, PYM-N1 respectively (Janke et al., 2004). Those promoter elements
were then inserted into the pESC-His, pESC-leu, or pESC-ura with BamHI and NdeI sites.

pET28A-FPS was built by Wu et al. (2006). pET28A-mFPSF112A was constructed by site directed mutagenesis with primers mFPS F112A F/R following the manufacture's instructions (Stratagene, San Diego, CA). Then mFPS gene was amplified and inserted into NdeI and SpeI sites on pESC-His-GPD vector and similar vectors but the GPD promoter is replaced with other listed promoter elements.

The fungal kaurene synthase, truncated grand fir abietadiene synthase, fungal kaurene oxidase, Arabidopsis kaurene oxidase, ABO and CYPB4, were synthesized with optimized codons for expression in yeast (Genescript, Piscataway, NJ), and inserted into pESC-Leu-GPD, or pESC-Ura-GPD as indicated in Figs. 3.7, 3.12, and 3.17. For mFPS and diterpene synthase inserted into one vector construct, pESC-Hls-GPD-mFPS was used as template to amplify with primers GPD-Nhel and TADH-Sall, then inserted into pESC-leu-GPD-ABS vector with Nhel and Sall sites. All other constructs with genes inserted into a single vector were built similarly.

3.5.6. Yeast fed-bath fermentation

Yeast diterpene producing strains were used for production of kaurene, abietadiene and kaurenoic acid by fed-batch fermentation. Yeast pre-cultures were prepared in Erlenmeyer flasks with optimized selection media at 23°C or 30°C as indicated with rotary shaking (180 rpm) for 2 days. The main culture medium (initial volume, 5.5 liters) in a 7.5-liter jar fermentor (New Brunswick Bioflo115) was seeded with a 200-ml aliquot of the pre-cultures. The temperature, agitation speed, flow rate of germ free air, dissolved oxygen, and pH were controlled at 23°C or 30°C as indicated, 600 rpm, 1 volume of air per volume of culture per minute, >40% saturation and pH controlled to 5.3 with 20% NaOH and 5% H₂SO₄. A total 1L 40% (w/v) glucose was fed at 15 g/h, once the initial starter culture glucose was depleted. Glucose was monitored using urinary glucose test strips available from common drug stores. During the fermentation, 2ml aliquots of culture were collected periodically to determine the amounts of terpene production and cell growth.

Kaurene and kaurenoic acid fermentation media contained 10 g Bacto yeast extract, 20 g Bacto peptone, 20 g glucose, 10 g KH₂PO₄, 0.5 g CaCl₂, 2 g NaCl, 3 g MgSO₄ per liter.
Abietadiene fermentation media contained 6 g Bacto-yeast nitrogen base (without amino acids), 1.4 g sigma yeast dropout solution (-his,-leu,-ura,-trp), 300 mg uracil, 150 mg L-tryptophan, 250 mg L-histidine, 20 g glucose, 10 g KH$_2$PO$_4$, 0.5 g CaCl$_2$, 2 g NaCl, 3 g MgSO$_4$ per liter.
Table 3.1. Primers used for the various molecular manipulations describe in materials and methods

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<thead>
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<th>Primer name</th>
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Table 3.1 (continued)
Chapter 4: Engineer Monoterpene Production in Yeast

4.1. Summary

Yeast do not harbor a specific GPP synthase (GPS), only a FPP synthase, encoded by the \textit{ERG20} gene, which catalyzes conversion of IPP and DMAPP to FPP without releasing GPP. Knockout mutations of the \textit{Erg20} gene in genetic backrounds of yeast able to meet their sterol requirement from exogenous sources (sue mutants – sterol uptake enhancement mutants) are lethal, probably because FPP is involved in protein prenylation and the formation of GGPP, necessary for dolichol and ubiquinone biosynthesis. To advance the ZX178-08 yeast line capacity for monoterpene production, two avian mutant FPSs, mFPSA116W and mFPSN144W, having significantly more GPS like activity than FPS activity, were integrated into yeast genome to replace the native \textit{ERG20} gene to create the new yeast lines ZXM116 and ZXM144, respectively. The most promising results were observed when a bifunctional strawberry linalool (monoterpene)/nerolidol (sesquiterpene) synthase gene, FaNES, was coexpressed in ZXM144 line on a plasmid vector, yielding 84.76±13.2 mg/L linalool, 20.54±3.8 mg/L nerolidol and no detectable farneol. In contrast, only nerolidol and farnesol, without any geraniol or linalool, accumulated when FaNES was expressed alone or co-expressed with mutant FPS having GPS activity in ZX178-08. These results indicate the successful shift in yeast metabolism from the biosynthesis of the 15 carbon intermediate FPP to its 10 carbon precursor, GPP.

4.2. Background

4.2.1. Monoterpene applications and uses

Monoterpenes consist of two isoprene units, hence consist of 10 carbon atoms, and are derived from GPP. Monoterpenes are found predominantly in plants where they are thought to function as defense agents (Hick et al., 1999), attractants for pollinators (Kessler et al., 2008), and may mediate plant-to-plant communication (Barkovich & Liao, 2001). More than 500 natural occurring monoterpenes have been identified, and many of these have commercial value as flavors, fragrances, pharmaceuticals, insect and antimicrobial control agents (Loza-Tavera, 1999). For instance, D-Limonene, which
possesses a strong smell of oranges, is commonly used in cosmetic products, such as hand cleansers, perfume, and shampoo (Bedoukian, 1983). Limonene is also widely used in food manufacturing, medicines, in cleaning solvents to remove oil from machine parts, and in some model airplane glues. Recently, limonene has also been suggested as a biofuel (Lohrasbi et al., 2010, Martín et al., 2010, Pourbafrani et al., 2010). Linalool, another common monoterpenes, is widely used as a scent in perfumed hygiene products and cleaning agents, such as soaps, detergents, and shampoos (Bedoukian, 1983). Linalool is also used as a flea and cockroach insecticide, and is the sole active ingredient in some mosquito repellent products. Nakmura et al. (2009) have also reported that aroma therapy with linalool can reduce stress in lab rats.

4.2.2 Conceptual framework for generating a GPP pool in yeast

Geranyl diphosphate, GPP, is the essential allylic precursor for all monoterpenes biosynthesis, and is not produced or produced in only very limited amounts in animals and microorganisms, including yeast cells. In fact, the yeast genome does not contain a geranyl diphosphate synthase (GPS) gene homolog. Instead, yeast harbors the gene ERG20, which encodes for a farnesyl diphosphate synthase, a multifunctional enzyme responsible for FPP biosynthesis from DMAP and IPP. Because of the apparent tight binding of GPP to the FPP synthase catalytic site, all the GPP generated from IPP and DMAPP by ERG20 is directly converted to FPP. Hence, the endogenous ERG20 enzyme in yeast is considered to be solely an FPP synthase. More specifically, if the intent is to generate monoterpenes products in yeast, the internal metabolism of yeast needs to be modified by building a GPP pool, and introducing an external appropriate monoterpenes synthase that can efficiently divert this GPP to monoterpenes biosynthesis as shown in Fig. 4.1.

There are many ways to build a GPP pool in yeast, at least conceptually. One would be to express a native plant specific GPP synthase enzyme in yeast with hopes a second enzyme, a monoterpenes synthase also introduced into the yeast, would be efficient enough to divert the GPP so produced to monoterpenes biosynthesis. A second means would be to mutate a robust FPS into a GPS-like enzyme and to introduce this into the yeast in a way that this prenyltransferase would yield sufficient amounts of FPP to meet this metabolic need in yeast, yet produce an excess of GPP. GPS belongs to the short chain prenyltransferase family of enzymes. This protein family includes GPS, FPS
(farnesyl diphosphate synthase), and GGPS (geranylgeranyl diphosphate synthase), and they all share a significant level of amino acid sequence similarity. Sequence alignment of various example sequences for these prenyltransferases has revealed five conserved amino acid sequence domains (Bouvier et al., 2005). Two important Asp-rich Motifs, FARM (First Asp-Rich Motif) and SARM (Second Asp-Rich Motif), both of which share the consensus sequence DDX$_{2-4}$D, X representing any amino acid (Bouvier et al., 2005). These two highly conserved Asp-rich motifs are important for catalytic function by supporting substrate binding. FARM has also been designated as the chain length determination region (Bouvier et al., 2005, Narita et al., 1999). A simple model for consecutive condensations of IPP by FPP synthase was illustrated by Ohnuma et al. (1996) and is shown in Figure 4.2. First, FPP synthase accepts IPP and DMAPP as substrates. After two consecutive condensations of IPP, the ω-terminus of the formed FPP reaches the amino acid at the fifth amino acid of the FARM domain (Ohnuma et al., 1996). If the side chain of this amino acid is large, further condensation is inhibited, resulting in formation of only FPP. On the other hand, when the side chain is smaller, the condensation continues to give longer products such as GGPP and HPP (Tarshis et al., 1996). Using the 3D crystallographic structure and site direct mutagenesis, mutant forms of FPS showed that the amino residue at the fourth and fifth position of the FARM block can serve as a steric hinderance to the allylic substrate binding site and prevent further product chain elongation (Bouvier et al., 2005). Directed mutagenesis of FPP synthase also generated mutant enzymes capable of further elongating the FPP as well. For example, mutation of phenylalanine at position 112 to alanine (F112A) of the avian farnesyl diphosphate synthase (FPS) results in a enzyme capable of producing geranylgeranyl diphosphate, while a F113S mutant yields geranylfarnesyl (C25) diphosphate as its dominant product, and even longer chain prenyl diphosphates when the double mutation F112A/F113S) was constructed (Tarshis et al., 1996).
Figure 4.1. Overall design for generating a yeast strain, ZXM144, capable of producing monoterpenes.

The new generated yeast line would have a dispensable sterol biosynthetic pathway, and include the substitution of the native FPP synthase $ERG20$ with a mutant avian prenyltransferase selected for its ability to catalyze the robust generation of GPP. The build-up of a GPP pool would provide the necessary precursor for the biosynthesis of monoterpenes compounds.
**Figure 4.2.** A schematic diagram of how the avian farnesyl diphosphate synthase is converted to a geranyl diphosphate synthase-like enzyme.

The simple model for consecutive condensations of IPP by FPP synthase was adapted from the models of Ohnuma et al. (1996) and Fernandez et al. (2000). FPP synthase first accepts IPP and DMAPP, then condenses these two intermediated to GPP. In the mutant FPS model, the chain length determining region is occluded by a larger size amino residue, making the binding pocket shallower and prohibiting the further elongation of GPP to FPP, resulting in the release of GPP as the dominant product. In the wide type FPS model, a second consecutive condensation with IPP could happen and form the FPP as product. Kinetic parameters were determined by Fernandez et al. (2000): Wt FPS: Km=0.87 µM for IPP, Km=1.10 µM for DMAPP, Km=0.37 µM for GPP; mFPS A116W: Km=8.4 µM for IPP, Km=44 µM for DMAPP, Km=440 µM for GPP; mFPS N144W: Km=6 µM for IPP, Km=100 µM for DMAPP, Km=260 µM for GPP.
The distribution of GPSs (GPP syntheses) is limited in nature with some functioning as homodimers, like the Arabidopsis GPS (Bouvier et al., 2005), tomato GPS (Van Schie et al., 2007), three Grand Fir GPSs (Burke & Croteau, 2002) and an orchid GPS (Hsiao et al., 2008). Others function as heterodimers, which requires interaction between two separate and distinct subunits for enzyme activity. Examples of the heterodimeric GPSs include that from Clarkia, snapdragon, and mint (Burke et al., 2004). The large subunits of heterodimeric GPS share high amino acid sequence identity (50%-75%) with other prenyltransferases, while the heterodimer small subunits share much lower sequence identity with other prenyl diphosphate synthases (22-38%) and lack any Asp-rich motif. Interestingly, it has been found that the interaction of a small subunit of GPS with a homodimeric GGPPS from Taxus canadensis and Abies grandis can functionally convert these enzymes into GPS like enzymes (Burke et al., 2004), suggesting that the heterodimeric small subunit plays an important role in controlling prenyl chain length.

Because heterodimeric GPSs function as a pair of proteins with different amino acid sequences, balanced and coordinated expression of the two different genes to obtain the final functional protein is necessary. For this reason, given our aim to engineer monoterpene biosynthesis, it appeared that engineering of a homodimeric GPS would be a better choice than trying to engineer a heterodimeric GPS. All the homodimeric GPS protein sequences currently described from Chlamydomonas (Chlamydomonas reinhardtii XM001691017 (GeneBank accession number)), Grand Fir (Burke & Croteau, 2002), grape (Vitis vinifera AJ243739), tomato (Solanum lycopersicum DQ286930), and Arabidopsis (Y17376, Bouvier et al., 2000) share high sequence similarity and contain the two asp-rich motifs. Exceptions to the sequence conservation within the homodimer group of GPS include the GPS from soybean (Glycine max EU399509), orchid (Hsiao et al., 2008) and a bark beetle Ips pini (Gilg et al., 2005), which lacks the conserved Asp-rich motif. The only homodimeric plant GPS without an Asp-rich motif is from Orchid and this enzyme exhibits dual prenyltransferase activity producing both GPP and FPP. It contains a putative active site EAEVE motif, which could provide a substitute binding site for Mg2+ and serve the reaction in a manner similar to the SARM (Hsiao et al., 2008).

Considering the low GPS enzyme activity observed in preliminary experiments with an Arabidopsis homodimeric GPS, we instead began work on evolving a GPS from FPS as described by Fernandez et al. (2000) for the avian FPS. The mutations of A116W and
N144W of avian FPS putatively reduce the size of the binding pocket, which was identified by molecular modeling (Fernandez et al., 2000). The depth of the pocket that binds the hydrocarbon chain of the allylic substrate determines the number of isoprene residues (IPP) that could be added to the growing chain. The mutants N144W and A116W, which have bigger amino acid side chains, would tend to narrow or constrict the substrate binding pocket, stopping chain elongation from GPP to FPP, thus converting FPS into a selective GPS. These two mutant A116W and N144W exhibit GPP: FPP selectivity of about 35 and 185 to 1 respectively, but they only have approximately 35% of the activity of wild type FPP in terms of DMAPP utilization (Fernandez et al., 2000).

Wu et al. (2006) reported that coupled over-expression of the avian FPS with sesquiterpene synthases in transgenic tobacco plants yielded about 30 µg/g FW sesquiterpene product, more than 1,000 times higher levels of sesquiterpene than in plants harboring only the synthase transgene. But, in constrast, over-expression of the Arabidopsis homodimeric GPS and a monoterpene synthase only yielded monoterpenes accumulation about 1/100th that for sesquiterpenes. This information too suggested that the Arabidopsis homodimeric GPS could be limiting for GPP production in those transgenic plants.

Altogether, the existing information pointed to the need for a more robust GPS sufficient to build up a GPP pool in yeast. And although the relative activity for the N144W and A116W mutants of the avian enzyme were slightly compromised, we considered the heterologous over-expression of these mutants in yeast as a viable means for building up the endogenous GPP pool and still providing the necessary FPP for viability. However, we also consider the buildup of a GPP pool in yeast as problematic without some means for down-regulating ERG20 and the conversion of any GPP to FPP that would be catalyzed by this enzyme. Moreover, because FPP is utilized in several biosynthetic processes, a direct knockout of ERG20 would be a lethal mutation. FPP is used in protein prenylation, and the formation of GGPP is necessary for dolichol (important for glycosylation reactions) and ubiquinone (important for electron transport) biosynthesis. Since the mutant avian FPS A116W and N144W mutants still maintained some FPP biosynthetic activity, we assumed that a direct replacement of ERG20 with either of the mutant avian enzymes could provide sufficient amounts of FPP for normal growth requirements, and could provide the necessary flux of carbon to GPP biosynthesis to support further monoterpenes biosynthetic engineering.
4.2.3 Enzymatic active monoterpane synthase

Monoterpene, the volatile compounds contained in essential oils, are widely applied in flavors or pharmacological agents. Interestingly, monoterpene synthesis can be carried out by colorless plastids, leucoplasts found in specialized secretary gland cells associated with leaves and stem trichomes or with the secretary cavities of citrus peel, as well as in the normal chloroplasts found throughout plants (Buchanan et al., 2002). Monoterpene synthase are responsible for the specific diversion of GPP to monoterpenes. Many monoterpane synthases have been isolated from essential oil producing species. Three monoterpane synthases (terpinene synthase, pinene synthase and limonene synthase) from lemon have been been metabolic engineering into tobacco to increase fragrance, which produced limonene as levels as high as 1.2 µg/g fresh weight sample every 24 hours (Lücker et al., 2001). Transgenic tobacco plants engineered by (Wu et al., 2006) for over-expression of an Arabidopsis GPS and a lemon limonene synthase accumulated upwards of 500 ng/g FW. Aharoni et al. (2003) observed that constitutively expressing a strawberry dual linalool/nerolidol synthase (FaNES1) in Arabidopsis resulted in linalool and its glycosylated and hydroxylated derivatives accumulating up to 13 µg linalool per day. FaNES1 is a multi-functional terpene synthase that can synthesize a monoterpane, linalool, from GPP and a sesquiterpene, nerolidiol, from FPP (Aharoni et al., 2004). Over-expression FaNES1 in yeast could thus serve as a measurement of the endogenous pools of GPP and FPP, and monitoring the endogenous shift from FPP to GPP accumulation in erg20 defective lines. Hence, we decided to use FaNES1 as a means for monitoring the success of our strategy to metabolic engineer yeast for monoterpane biosynthesis.

4.3. Results

4.3.1. Mutating a FPS into a GPS

Our attempts to engineer elevated GPP levels in yeast using known plant geranyl diphosphate synthases were unsuccessful. In fact, all the known GPS enzymes appear to possess poor catalytic efficiencies for GPP biosynthesis. We therefore decided to evolve GPS activity from the very robust avian FPS according to (Fernandez et al., 2000). The mutants A116W and N144W of the avian FPS are predicted to restrict the size of the active site pocket, stopping chain elongation from GPP to FPP, and thus
converting FPS into a more selective GPS while maintaining relatively good catalytic efficiencies, up to 35% of the wild type enzyme.

Wide type avian FPS was subcloned from a construct built by Wu et al. (2006) into Pet28a vector under the control of an IPTG inducible T7 promoter. The mutant FPSs were generated with the primers described by Fernandez et al. (2000) via site-directed mutagenesis with pET28a-FPS serving as the template. Then the constructs were transformed into E. coli BL21. After induction by IPTG, the mutant FPS lines gave similar protein levels to the wide type FPS as shown by Coomassie Blue stained SDS-PAGE gels of total protein extracts from the transformed lines of E. coli (Fig. 4.3A).

The prenyltransferase selective enzymatic activities of mutants FPS toward synthesis GPP relative to wild type FPS were identified by the assay with DMAPP and IPP as substrate and cell-free extract lysate to produce GPP or FPP. When radioactive substrates were used, the reaction solution was extracted by butanol and aliquots of the extract counted in a liquid scintillation counter to gain a measure for the total prenyltransferase (GPP and FPP) activity. The mutant enzymes possess approximately 70% or better of the catalytic activity as the wild type enzyme. When non-radioactive substrates were used, after the reactions were incubated, alkaline phosphatase was added to hydrolyze the prenyl diphosphates (GPP and FPP) to the corresponding alcohols GOH and FOH, and then the products could be separated and quantified by GC-MS as described in Fig.4.3B & C. For wild type FPS when supplied with IPP and DMAPP as substrates, it produced both GPP and FPP, while the two mutants mFPSA116W and mFPS-N144W only produce GPP in our cell-free cold assay system. mFPSA 116W gave about 80% product yield and mFPS-N144W gave 67% product yield relative to the wild type enzyme. Based on the studies of Fernandez et al. (2000), these two mutant A116W and N144W should exhibit GPP: FPP selectivity of about 35 and 185 to 1 respectively. We were not able to confirm these selectivity ratios in part because of the sensitivity of our instrumentation. Nonetheless, we decided to test both of these two mutants in our yeast system.

4.3.2. Replacement of yeast FPP synthase gene, ERG20, with the avian mFPS A116W and N144W through homologous recombination

Because we did not know if the residual FPS activity for either of the two mFPS would be sufficient to complement an erg20 knock out in terms of supplying FPP, we designed
a two-step replacement strategy (Fig. 4.4). First, an algal, *Botryococcus braunii*, FPS (bFPS) was inserted into a *URA3* expression vector and introduced into the yeast ZX178-08 line. This was done to assure that a knockout mutation in the *ERG20* gene would not be lethal. Then we went about the process of designing and constructing site-specific recombination cassettes bearing the mutant avian FPSs between 5' and 3' sequences of the *ERG20* locus. Integration of these cassettes into the yeast genome would remove the intact *ERG20* gene and replace it with the various mutant FPS genes. To monitor these integration events, yeast were grown in complete medium for 48hr, then plated onto selection medium minus Leu. About twenty single independent yeast transformants grew and were picked for further confirmation of recombination. Genomic DNA isolated from the transformants and control yeast line ZX178-08 were used as template for PCR reactions with four sets of primers to verify the correct gene replacement of the target *ERG20* as described in Fig 4.5. Transformant #4 was negative in PCR reactions A, which is specific for the *ERG20* gene, yet positive in PCR reactions B, a reaction specific for the avian mFPS gene. Transformant #4 was also gave positive and correct sized DNA fragments in PCR reactions C and D, also consistent with the proper integration of the mFPS-LEU2 constructs into the *ERG20* locus.

The next step was to evict the plasmid vector harboring the bFPS gene. If the mFPS genes integrated into the genome were sufficient in meeting the cell’s demands for FPP, then the yeast lines without the bFPS construct would be viable and afford the yeast lines with a pool of GPP for monoterpenes biosynthesis. When positive yeast transformant # 4 was replica plated onto minimal medium containing 5FOA (5-fluoroorotic acid) to counter-select for lose of the *Ura3* containing episomal vector, yeast line ZXM116 was obtained. 5-FOA is nontoxic to yeast, but in strains expressing a functional *URA3* gene, 5-FOA is converted to the toxin 5-flourouracil. In an identical manner, yeast strain ZXM144 was development by replacement of the *ERG20* gene with the mFPS N144W gene. Both of these yeast lines produced a significant amount of geraniol as determined by GC-MS, and had a distinctive rose-like scent which differed from the common scent of yeast.
Figure 4.3. Characterization of prenyltransferase activities.

A) FPS and mutant FPS genes were expressed in *E. coli* BL21 (DE3) cells, total proteins extracted, size separated by SDS-PAGE and the gel stained with the general protein stain Coomassie Blue. Total proteins from *E. coli* harboring the expression vector only (no foreign gene inserts) (lane 2), or the total proteins from *E. coli* expressing wild type FPS gene, the FPS A116W mutant or the FPS N116W mutant genes (lanes 3, 4, and 5 respectively). B) A cartoon depiction of the assay used to qualify the reaction products of the different prenyltransferases as measured in the GC-MS assays. C) Actual GC-MS assessment of the reaction products generated by the indicated bacterial extracts incubated with IPP and DMAPP as substrates. While wild type FPS generated both GPP and FPP in these in vitro assay conditions, both mutants appear to only produce GPP. Mutant FPS A116W gave approximate 80% product yield relative to the wild type enzyme, while mFPSN144W gave approximately 67% product yield. The retention times and mass spectra for the indicated GOH and FOH peaks matched authentic standards.
Figure 4.4. A schematic diagram of the overall approach to generate new yeast strains with the ability to accumulate high levels of GPP, a key precursor for monoterpenoid biosynthesis.

ZX178-08 is a yeast line that accumulates high levels of FPP and has a dispensable sterol biosynthetic pathway because of a knockout mutation in its squalene synthase gene, ERG9. ZX178-08 hence served as the initial parental line for generating a strain that accumulates GPP, a key intermediate in monoterpenoid metabolism, rather than FPP. To limit FPP biosynthesis in ZX178-08, a knockout mutation in the native FPS gene, ERG20 was desired. However, because a complete knockout and elimination of all FPP would be a lethal mutation, we sought to replace the ERG20 gene with a prenyltransferase yielding some FPP, but mostly GPP when expressed. Two mutant FPS genes, referred to as A116W and N144W for the particular amino acid modification in each, encode for prenyltransferases that have robust GPP biosynthetic activity, but still have some capacity for FPP biosynthesis. To replace the native FPS synthase gene ERG20 with these mutant forms required a two-step process. First, to assure a supply of FPP, the yeast were engineered with an algal FPP synthase on an episomal vector. Then the mFPS genes were flanked by 5' and 3' sequence around the ERG20 gene, which facilitates the replacement of the ERG20 gene by homologous recombination. After selection for independent recombinant colonies each of the transformants can be PCR screened for replacement of the ERG20 gene. The positive ERG20 replacement lines can then be replica plated onto medium containing 5-FOA to select for loss of the bFPS plasmid. The resulting yeast lines are named ZXM116 or ZXM144.
bifunctional monoterpenesesquiterpene synthase gene, FaNES1, can be introduced into the new ZXM yeast series to divert any excess GPP and FPP to monoterpenesesquiterpene biosynthesis, respectively. To further enhance monoterpenesesquiterpene biosynthesis, the introduction of an extra copy of the mFPS can also be added to the episomal vector.
4.3.3. Measuring the GPP and FPP pools in yeast by over-expression of the FaNES1 gene

To evaluate the utility of the new yeast lines ZXM116 and ZXM144 for monoterpene production, we first examined their accumulation of geraniol, the dephosphorylated product from GPP while harboring an expression vector, Xhis, without any inserted monoterpene synthase gene. The rationale for this was that we would ultimately want to introduce a monoterpene synthase into these lines and hence wanted to establish the background levels for their substrate, GPP. While no geraniol was detectable in cultures of the parental yeast line BY4741 or ZX178-08, ZXM116 containing the Xhis vector accumulated 1.33±0.57 mg/L geraniol and 34.66±5.66 mg/L farnesol, and ZXM144-Xhis accumulated 2±1.1mg/L geraniol but no farnesol. The accumulation of both geraniol and farnesol in the yeast line harboring the A116W mutant FPS and essentially only geraniol accumulation in the yeast line containing the N144W mutant FPS is consistent with the observations of Fernandez et al. (2000), who noted that the N144W mutant exhibited a much greater GPP synthase activity relative to FPP synthase activity than the A116W mutant.

We next sought to determine how available the accumulating GPP pool might be for monoterpene synthase. For this purpose, we chose to over-express the strawberry FaNES gene, which encodes for a bifunctional terpene synthase (Fig. 4.6). If provided with GPP, the enzyme would convert this substrate to linalool. If provided with FPP, the NES enzyme catalyzes its conversion to nerolidol. Thus, over-expression of the NES enzyme activity could be used to measure, simultaneously, the available GPP and FPP pools. When the FaNES gene was expressed in the parental yeast line ZX178-08, nerolidol accumulated up to 140 mg/L over a 10 day culture period, but no linalool was detected (Fig. 4.7). Even when the avian A116W mutant FPS was included with the FaNES gene on the expression vector, no linalool was detected.

The FaNES gene was then introduced into the ZXM116 and ZXM144 yeast lines in combination with extra copies of the mutant FPS also included on the expression vector (Fig. 4.8). Small-scale cultures of transformants harboring the various plasmid constructs were then propagated for 6 days prior to examining the cultures for their terpene profiles. ZXM116 with just the expression vector (empty vector control) accumulated 1 to 2 mg/L of geraniol and in excess of 35 mg/L of farnesol. When the FaNES gene was introduced into this line, a slight decline in the amount farnesol accumulation was noted with only a
very modest accumulation of linalool and nerolidol. When an extra copy of the mutant avian FPS was included, either the A116W mutant or the N144W mutant, linalool and nerolidol levels increased 10- to 20-fold. A somewhat similar trend was noted for the ZXM144 line, except that the level of farnesol and nerolidol were substantially lower. Similar trends in chemical accumulation were also observed when the ZXM lines were grown in shake flask cultures and when harboring extra copies of the avian mutant FPS gene (Fig. 4.9). That is the greatest level of monoterpenes (linalool) accumulation was observed with the ZXM144 line co-expressing the FaNES gene and extra copies of mFPSN144W gene on episomal plasmid, with 84.76 ±13.2 mg/L linalool, 20.54±3.8 mg/L nerolidol, less than 1mg/L geraniol and no detectable farnesol observed over the 14 days of culturing.
Figure 4.5. PCR screening for replacement of the ERG20 gene with mutant FPS-LEU2 constructs.

DNA isolated from 6 independent colonies was used as PCR template with four sets of specific primers as illustrated in panel A. If the ERG20 gene (1059bp) was replaced by a mutant FPS, the amplification products expected in PCR reactions B, C, D are 1137bp, 2304bp, and 5580bp size fragments, respectively, but no amplification product is expected from the A reactions. With ZX178-08 genomic DNA as control template, only amplification products from PCR reactions A & D are expected, and no product is expected from amplification reactions B or C.
Figure 4.6. The strawberry FaNES1 gene encodes for a bifunctional enzyme that can use either GPP or FPP as its substrate, and can hence serve to measure the available GPP and FPP levels in vivo.

Strawberry FaNES1 is a bifunctional terpene synthase involved in monoterpene (C10) and sesquiterpene (C15) biosynthesis (Aharoni et al., 2004). FaNES catalyzes the conversion of geranyl diphosphate (GPP) into S-linalool and farnesyl diphosphate (FPP) into (3S)-E-nerolidol. The kinetic parameters for both activities are: Km=8.1 µM for FPP; Km=29.0 µM for GPP; Vmax=2.3 nmol/h/µg enzyme toward GPP; Vmax=3.0 nmol/h/µg enzyme toward farnesyl diphosphate (UniProtKB/Swiss-Prot: P0CV94.1). The bifunctional character of FaNES1 suggests that it can be used to measure GPP and FPP pools when expressed in yeast.
Figure 4.7. Expression of the strawberry FaNES gene in the parental yeast strain ZX178-08 only yields sesquiterpene products.

Previously developed ZX178-08 line has the dispensable sterol biosynthesis pathway and yeast squalene synthase (ERG9) knock out, accumulated high level of FPP, which can be utilized as the precursor for engineering sesquiterpene and triterpene. To test the capability of engineering monoterpene biosynthesis into ZX178-08, yeast expression vectors harboring FaNES alone or plus mutant FPS gene having GPS activity (Fernandez et al., 2000) were transformed into ZX178-08 yeast line, and then grown for 10 days prior to chemically profiling them for their chemical constituents by GC-FID and quantifying the levels of terpene accumulation in transgenic yeast. Terpene production and cell growth over 10-day shake flask culture are shown in panel A. No detectable geraniol or monoterpene related compounds accumulated, only farnesol and nerolidol were readily detected. GC was used to identify geraniol, linalool, farnesol, nerolidol and external standard cedrene from yeast extracts in comparison to authentic standards (B).
Figure 4.8. The expression of the strawberry FaNES gene alone and in combination with the avian mutant FPS in the ZXM yeast lines.

Yeast strains ZXM116 (A) and ZXM144 (B) were transformed with a vector control plasmid Xhis, this same vector with the FaNES1 only (Xhis-tFaNES), or the FaNES1 gene plus an extra copy of the mutant FPS as Xhis-116-tFaNES or Xhis-144-tFaNES. Yeast lines were grown as test tube shake cultures in 3 mL of SCE-Leu-His media containing 40 µg/ml of ergosterol for 6 days prior to sampling the cultures. Hexane extracts were concentrated and aliquots subjected to GC-FID to quantify linalool, geraniol, nerolidol and farnesol compound levels, relative to the external standard cedrene. The production level represents the average production from five independent single colonies.
Figure 4.9. Terpene accumulation in ZXM series yeast cultures over a 14 day period in shake flask cultures with selection medium.

Terpene accumulation and cell growth in yeast transformant lines ZXMO116 expressing Xhis-116-tFanes (A) or Xhis-144-tFaNES (B) and ZXMO144 expressing Xhis116-tFanes (C) or Xhis-144-tFaNES (D) were quantified and documented over 14 day culture.
Aliquots of hexane extracts prepared from the culture samples at the indicated time periods were subjected to GC-FID analysis and the linalool, geraniol, nerolidol and farnesol levels quantified relative to the external standard cedrene. Data points represent the average for 3 independent cultures. Panel E is an example GC chromatography of an extraction prepared from ZXM144 line expressing mutant mFPS N144W along with FaNES. Compounds were identified on the basis of their mass spectrum and retention time in comparison to authentic standards.
4.4. Discussion

Compared with engineering sesquiterpene biosynthesis and accumulation in *E. coli* or *S. cerevisiae*, much less progress in the metabolic engineering of monoterpenes has been achieved. There are probably many reasons for this, including less necessity for alternative production platforms when there are convenient natural sources already. Nonetheless, one of the other reasons monoterpenes lagged behind other classes of terpene is because most microbial platforms do not readily produce the key intermediate GPP, which is essential to build monoterpenes, and there is relatively little in depth information available about the GPP synthase enzymes and the means for engineering these enzymes for high level GPP production are rare.

Oswald et al. (2007) worked on evolving a GPS from FPS. The *erg20*-2 mutant yeast line is a leaky mutant which contains a K → E mutation at 197, leading to a 14-fold decrease in FPS activity (Blanchard & Karst, 1993). The *erg20*-2 strain also had the unusual property of accumulating geraniol. When in vitro enzyme assessments were done, the wide type ERG20 enzyme was shown to produce about 75% FPP and 25% GPP, while the *erg20*-2 mutant produced only 25% FPP, but 75% GPP (Oswald et al., 2007). Using 3D structure model for the yeast FPS, the K197E mutation could be rationalized as causing a reduction in the binding pocket size, which in turn could prevent the successive elongation of the bound GPP with a second condensation with IPP. In the wild type FPS, lysine 197 would bind tightly with GPP and allow the next head to tail condensation with IPP to form FPP (C15), while glutamic acid at position 197 of the mutant yeast FPS narrows the reaction pocket of yeast enzyme, preventing the next elongation to FPP. Overexpression of a sweet basil GES (geraniol synthase) in the *erg20*-2 mutant yeast line lead to the monoterpenol geraniol being excreted into the growth medium (500 µg/L). Fischer et al. (2011) went on to further improve the *erg20*-2 mutant by subjecting the K197 position to other amino acid substitutions. The major improvement achieved was 5 mg/L of geraniol when GES was over-expressed in this yeast line (Fischer et al., 2011).

Similar work has been carried out with a bacterial FPP synthase, IspA, and attempts to convert this enzyme into a GPP-like synthase. A mutation in Bacillus IspA, S82F, resulted in a distribution of GPP/FPP of 27:1, clearly indicating the importance of this site in the product specificity of the enzyme (Bouvier et al., 2005, Koyama et al., 1995). An
analogous mutation of E. coli IspA enzyme, S81F, was also used to engineer monoterpenes production to 0.42 µg/OD600/L/h (Reiling et al., 2004).

Overall, the monoterpene yeast lines developed here with co-expression of the bifunctional enzyme FaNES along with mutant prenyl transferase, have resulted in the highest levels of monoterpenes produced in microbes yet reported. This was created in large part by the generation of mutant FPS enzymes that afford sufficient amount of FPP to be produced to meet the physiological needs of the cell, plus diversion of excess carbon to the biosynthesis of GPP. The mFPS N144W mutant, for example, exhibited greater GPP selectivity than mutant mFPS A116W in vivo, consistent with the in vitro findings of Fernandez et al. (2000). In contrast, when the ZXM144 line harbored the FaNES gene plus extra copies of mFPS N144W gene on a plasmid vector, linalool levels in excess of 80 mg/L in shake flask culture was achieved, nearly 25 to 50% the level sesquiterpene accumulation observed earlier in Chapter 2. Our newly generated yeast lines cultures also have a very prominent rose-like scent coming from volatile monoterpenes. These indicate a continuous loss during cultivation, and the productivity we measure as the monoterpene content in the cultures is certainly an under-estimation of the real productivity. Additional efforts to account for head gas losses will be necessary to fully appreciate the productivity of our metabolic engineered yeast lines.

4.5. Materials and method

4.5.1. 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. YPD media for growing yeast without selection consisted of 1% Bactoyeast extract, 2% Bacto-peptone, and 2% glucose. YPDE media was YPD media supplemented with ergosterol (40 mg/L) for ergosterol dependent lines. Ergosterol. Ergostrol stocks were 10 mg/mL in 50% Triton X-100, 50% ethanol and kept at -20°C. Minimal media, SCE (pH 5.3), contained 0.67% Bacto-yeast nitrogen base (without amino acids), 2% dextrose, 0.6% succinic acid, 0.14% Sigma yeast dropout solution (-his,-leu,-ura,-trp), uracil (300 mg/L), L-tryptophan
(150 mg/L), L-histidine (250 mg/L), L-methionine (200 mg/L), L-leucine (1g/L) and 40 mg/L. Selection media was prepared similarly except without supplementing the media with the indicated reagent based on the yeast auxotrophic makers. All solid media plates were prepared with 2% Bacto-Agar.

### 4.5.2. Yeast transformation and culture performance

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. About 1 µg of plasmid or about 5 µg of linearized DNA was used per transformation and followed by selection on agar plates of SCE medium lacking specified amino acids for the auxotrophic markers at 30°C. Variable numbers of independent colonies were subsequently picked and used to start 3ml cultures in minimal media for isolating genomic DNA or characterize their terpene production capacities. To verify the correct homologous recombination of replacement of ERG20 more than 20 independent colonies were picked, cultured and prepared for genomic DNA by using YeaStar™ Genomic DNA Kit (Zymo Research, Orange, CA). Four sets of primers PA (ERG20F, ERG20R), PB (FPSF, FPSR), PC (LEU2 NotIF, Yeast X106918R), PD (Yeast X104229F, Yeast X106918R) as listed in table 4.1 were used to screen the positive replacement of ERG20. For terpene production quantification, aliquots of yeast cultures were analyzed by GC-MS after 6 days of incubation at 23°C with shaking. Cultures exhibiting the highest terpene production levels were chosen for further time course studies and archived as glycerol stocks at -80°C. Selected lines were characterized for cell growth and terpene production using 30 mL shake flask cultures. Starter cultures grown to saturation in minimal media were inoculated into 30 mL SCE media and 1 mL aliquots withdrawn at every other day intervals for 10–15 days. Cell growth was monitored as the change in optical density at 600 nm every two days, using appropriate dilutions for cultures at later stages of growth. Terpene production was determined by GC-MS similar to the initial screening method.

### 4.5.3. GC-MS detection and quantification of terpenes

To determine terpene accumulation levels, aliquots of cultures grown for 6 to 12 days were extracted with hexane and aliquots evaluated by GC-MS. In general, to 1 volume of culture, 1 volume of acetone was added and mixed vigorously and left on bench for 15min to lyse the cells. The sample was then allowed to incubate at room temperature
for 10 min before adding 1 volume of hexane containing a known amount of cedrene external standard. The mixture was again mixed vigorously, then centrifuged in a clinical centrifuge for 5 min at maximum speed. The upper organic layer was collected and when necessary, concentrated under a N₂ stream to 100 µl. An aliquot of the organic phase (1 µl) was then analyzed by GC-MS with a Varian CP-3800 GC coupled to a Varian Saturn 2200 MS/MS (Varian Medical Systems) using a Supelco SLB-5ms fused silica capillary column (30 m x 0.25 mm x 0.25 µm film thickness, Supelco). The initial oven temperature was set at 60 °C for 2 min, ramped to 200 °C at 8 °C/min, and then ramped to 300 °C at 20°C/min and held for 1 min more. Linalool, geraniol, nerolidiol and farnesol levels were calculated relative to the cedrene external standard.

4.5.4. Construction of the farnesyl diphosphate synthase erg20 Knockout mutation

Figure 4.10 illustrates the general strategies used to construct the various expression vectors. The yeast GPD promoter (Pgpd) was amplified from the PYM-N14 plasmid described by (Janke et al., 2004) using the primers PTEF-PGDF and PGPD-EcoRIR. The yeast TEF1 promoter (Ptef1) was amplified from the PYM-N18 with PTEF-PGDPR and PTEF-BamHIR primers (Janke et al., 2004). After purification 200 ng Pgpd and Ptef was mixed and used as template to run second PCR with primers PGPD-EcoRIR and PTEF-BamHIR to assembly these two promoters together for building the pesc-X series vectors containing two strong constitutive divergent promoters for expression two genes in one single vector. The obtained two GPD and TEF promoter fusion product was inserted into the pESC vector series digested with EcoRI and BamHI to replace the original GAL1/10 promoters to construct the new X series vector. The resulting plasmids were named Xhis, XUra, XLeu.

The LEU2 and bFPS gene were cloned into NotI and SpeI sites of XUra to obtain the yeast expression vector XUra-Leu and XUra-bFPS. Then the mutant FPS genes were amplified from pet28A-mFPS116 or Pet28A-mFPS144 with primers (FPS BamHIF and FPS XmaIR), and inseted into XUra-Leu multiple cloning sites digested with BamHI and XmaI. The resulting vector was used as template to amplify the fragment A harboring mFPS gene and Leu2 selection marker with primers ERG20L-TCYC2F and TADH-ERG20R2 having homologous sequences to Erg20 with TCYC or TADH as illustrated in Fig4.8.
Figure 4.11 illustrates the vectors and experimental strategy for replacing the ERG20 gene with the different mutant FPS genes. The primers (Yeast X-104429F and Yeast X-106818R) as list in Table 4.1 were used to amplify the farnesyl dipiphosphate synthase ERG20 (on chromosome X from coordinates 105014 to 106072) along with the surrounding 612 bp up and 746 downstream regions in the yeast genome from the yeast ZX178-08 genomic DNA by using Takara high fidelity Primestar taq polymerase. The obtained PCR fragment was gel purified, A tailed and ligated into the pGEM-Teasy vector (Promega, WI) by following the manual. The obtained vector was used as template to run second PCR with primers Erg20L-TCYC2R and ADH-Erg20RF2 to obtain PCR fragment B containing 30bp homologous region to ERG20 surrounding region plus flanking with 30bp homologous to TCYC and TADH as illustrated in Fig 4.8.

To build the ERG20 replacement construct, PCR fragment A and B were treated with DpnI to digest the template plasmid, and then assembled together with Gibson Assembly Kit (New England Biolab, Ipswich, MA). The resulting construct was used as template to amplify fragment C as shown in Fig. 4.11 which contained the homologous sequence to ERG20 surrounding regions, mFPS and Leu2 selection marker. These PCR fragments were than transformed to the ZX178-08 yeast line harboring XUra-bFPS vector following the standard protocol but without growth in YPDE rich medium for 48 hr before selected on minus Leucine plates. Over twenty independent single colonies were picked for PCR confirmation of recombination with four primer sets listed above, as well as geranyl and farnesol production analysis.

Yeast lines with correct recombination were replica plated on SCE-Leu plates containing 5-FOA to select for loss of the XUra-bFPS vector, and the resulting yeast lines named ZXM116 (ERG20 replaced by mFPSA116W) or ZXM144 (ERG20 replaced by mFPSN144W).

**4.5.5. Expression of the FaNES and extra copy mFPS in yeast**

Total strawberry total RNA was extracted from mature strawberry with QIAGEN RNeasy plant mini kit (QIAGEN, Valencia, CA), and double strand cDNA was synthesized according to the method described in Stratagene. Twenty nanogram of double-strand cDNA was used as template for PCR with primers tFaNES NotIF, and tFaNES SpeIR. The PCR fragment was inserted into Xhis-Leu-mFPS116 and Xhis-Leu-mFPS144 vectors digested with NotI and SpeI to replace the Leu2 selection marker. The resulting
vectors were named Xhis116tFaNES and Xhis-144-tFanes. Xhis-tFaNES construct was built similarly, but the tFaNES fragment was inserted into Xhis vector digested with NotI and SpeI. Yeast lines transformed with these constructs plus Xhis control were then evaluated for their production of the linalool, geraniol, nerolidol and farnesol as a measure of the available of intermediates of the mevalonate biosynthetic pathway for the biosynthesis of monoterpenes and sesquiterpenes.

4.5.6. Characterization of prenyltransferase activities.

4.5.6.1 Functional protein expression in E.coli

Pet28A-FPS was built by Wu et al. (2006). Pet28A-FPS116 and Pet28A-FPS144 were constructed by site directed mutagenesis with primers mFPS A116WF/R and mFPS144WF/R following the manufacture instruction described by stratagene. E. coli BL21(DE3) cells are transformed with the resulting recombinant plasmids, as well as an empty pET vector for use as control. Single positive bacteria colonies, confirmed by DNA sequence, were used to inoculate 10 mL LB medium with 100 µg/ml of kanamycin, and cultured overnight at 37°C. One mL of these cultures was then inoculated into 100 mL LB medium containing 100 µg/ml of kanamycin to grow continuously at 37°C until OD reached 0.5-1.0. Transcriptional expression of the recombinant genes downstream of the T7 promoters in the pET vectors were then induced by the addition of 1mM isopropyl-1-thio-b-D-galactopyranoside (IPTG), and the cultures grown at room temperature overnight.

4.5.6.2. Preparation of E. coli lysates and purification of recombinant proteins with Ni column

One hundred mL of overnight IPTG-induced cultures were harvested by centrifugation at 4°C and resuspended in 4 mL of lysis buffer (80 mM postassium phosphate buffer, Ph7.0, 10mM Meta-bisulfate, 15mM MgCl₂, β-mercaptoethanol,). Cells were disrupted by sonication using a microprobe operated at 60% power with 20s treatments followed by cooling for 1 min for a total of 8 treatments in a cold room. Cell debris was cleared by centrifugation at 13,000g for 30 min at 4°C. The resulting supernatants were used for enzyme assays as well as protein purification. For purification, the cleared lysates were diluted to 8 ml with 2X binding buffer then run through a nickel-activated His-tag column. The column was washed with 6 mL of binding buffer containing 20mM Tris-HCl, pH 7.9,
500 mM NaCl, 5mM imidazole, followed by increasing concentrations of imidazole in the same buffer up to a 500 mM imidazole concentration. One ml fractions were collected and used for enzyme assays.

4.5.6.3. Detection of prenyltransferase activity

Prenyltransferase assays were performed in a final volume of 50 µl containing 20 µM [1-14C]-IPP (55 mCi/mmol), 20 µM cold IPP and 40 µM DMAPP in assay buffer (50 mM Tris-Hcl, pH 7.0, 10 mM MgCl2) with crude or purified proteins and incubated for 1 hr at 30°C. After the reaction is over, 200 µl of saturated NaCl was added to the reaction solution and 1 ml of water-saturated 1-butanol was added, vigorous mixed for 15 sec, and after a short spin, 250 µL of the 1-butanol phase was counted in a liquid scintillation counter as a measure of GPP plus FPP biosynthesis.

To evaluate the specific activity of GPP vs FPP, after the reaction is inactivated by chilling on ice, 6 µl of 10X alkaline phosphate buffer and 4 µl of Promega alkaline phosphatase (1unit/ ul) were added. After an incubation at 37°C for 2hr, 60 µl of saturated NaCl and 200 µl hexane were added, vigorous mixed mixed for 15 sec, and after a short spin, 100 µl of hexane were collected and mixed with 5ul of 100 ng/ul of cold geraniol and farnesol as carrier. After concentration under nitrogen gas to 30 µl, samples were separated on reverse phase TLC plate developed with acetone/H2O (9:1) and visualized by iodine vapor; the zones corresponding to those of GOH and FOH were scraped separately and counted by liquid scintillation counter.

4.5.6.4. Prenyltransferase product analysis

For product analysis, one mL reactions were performed under the same conditions as the enzyme assays, except 40µM cold IPP but no radioactive IPP was used. After an overnight incubation at 30°C, 110µl of 10x alkaline phosphate buffer and 10 units of Promega alkaline phosphatase were added to hydrolyze the prenyl diphosphates to their corresponding alcohols. The reaction mixture were incubated at 37°C for 3hr, and then extracted with 3ml of hexane with vigorous shaking for 15s. After a brief centrifugation, the hexane extract was concentrated under nitrogen gas and then analyzed by GC-MS.
Yeast expression vectors designed for the strong, constitutive expression of the bFPS, mFPS-A116W, mFPS-N144W, FaNES1, and LEU2 selection genes directed by the GPD promoter (Pgpd) or the TEF1 promoter (pTEF), and termination provided the ADH terminator sequence (T-ADH) or CYC terminator sequence (T-CYC) along with His and Ura3 auxotrophic markers.

**Figure 4.10.** Schematic depiction of the constructs assembled to replace the ERG20 gene (A and B) and to engineer monoterpene metabolism (C and D) in yeast.
Figure 4.11. A schematic diagram of the steps required to build the various expression plasmids, their use in replacing the ERG20 gene, and identification of the primer sets (PA, PB, PC, and PD) used to verify homologous recombination at the target locus.
Table 4.1. Primers used for the various molecular manipulations describe in materials and methods.

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<thead>
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### Table 4.1 (continued)

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<tr>
<td>Yeast X 106918R</td>
<td>CTTACATACCAAGGTAGAAATGTTAAAGATA</td>
</tr>
<tr>
<td>FPS F</td>
<td>ATGCAGCCCCCATCATCATCATAAAGAG</td>
</tr>
<tr>
<td>FPS R</td>
<td>TCATTTCCTGGCTTTGTAGATCTTCTG</td>
</tr>
<tr>
<td>ERG20 F</td>
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<tr>
<td>ERG20R</td>
<td>CTATTTTGCTTCTTGTAAACTTTG</td>
</tr>
<tr>
<td>FPS-BamHIF</td>
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</tr>
<tr>
<td>FPS-XmaI R</td>
<td>GGGCCCGGGTCATTTCTGGCGTTTGAGATC</td>
</tr>
<tr>
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</tr>
<tr>
<td>tFaNES SpeI R</td>
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Chapter 5: Engineer triterpenes metabolism in yeast

5.1. Summary

To eliminate the native squalene catabolism in yeast and to extend the application of our novel yeast line for triterpene production, insertional mutants into \textit{ERG1} gene (coding squalene epoxidase) were generated. When BSS (squalene synthase) was introduced into ZXB line, having an extra copy of tHMGR integrated into the \textit{ERG1} deletion region, the resulting yeast transformant accumulated production of 297.7 mg/L squalene and 70.24 mg/L farnesol in shake flask conditions.

5.2. Introduction

Triterpenes are compounds containing 30 carbon atoms synthesized from isoprene units assembled in the cytoplasmic compartment by the mevalonate biosynthetic pathway. Three isoprene units are polymerized into 15 carbon units referred to as farnesyl diphosphate, FPP, and two FPP molecules are condensed in head-to-head reactions to form squalene. Squalene is then converted to 2,3-oxidosqualene by squalene epoxidase, and various oxidosqualene cyclases catalyze the cyclization of 2,3-oxidosqualene to produce a wide variety of four and five ring structures that can be further decorated by hydroxylation, acylation, aroylation, methylation, and glycosylation. Triterpenoids play diverse functional roles in all eukaryotes. In plants, when triterpenes are directed towards 4-ring structures and particularly sterols, these compounds play essential roles in plant growth by serving as structural components in cell membranes, influence the lipid bilayer fluidity, as in regulatory roles serving as growth regulators or hormones. Brassinolides represent a particular important class of growth regulators in plants. Other triterpenoids fall into the classification of specialized metabolites, compounds that serve in plant specific manners to mediate interactions between plants and their environment. Avicins are triterpenes produced in the roots of oat species that provide protection against microbial pathogens (Haridas et al., 2001a, Haridas et al., 2001b). In some solanaceous plants, specific triterpenes accumulate in aerial parts of the plants and serve as feeding deterrents to insect pests (Itoh et al., 1977). More recently, triterpenoids have been advanced for their utility as high quality biofuels. Botryococcene, a triterpene hydrocarbon oil, is the major oil constituent of \textit{Botryococcus}
*braunii*, a colony-forming green algae, that accumulates up to 30-80% of this algae’s dry weight. The botryococcene oils have also attracted significant research interest because these triterpene oils from a single organism have been claimed to contribute up to 1.4% of the total hydrocarbon content in oil and coal shale deposits (Moldowan & Seifert, 1980, Okada et al., 1997, Derenne et al., 1997, Glikson et al., 1989), and can be readily converted to high quality combustible fuels such as gasoline, jet fuel and diesel, under standard hydrocracking/distillation procedures. Niehaus et al. (2011) recently identified the unique mechanisms for botryococcene biosynthesis in *Botryococcus braunii* and functional characterized the SSL1 (squalene synthase-like protein 1) and SSL3 (squalene synthase-like protein 3) enzymes as those responsible for catalyzing the two separate step reactions converting two FPPs to botryococcene. Another common eukaryotic triterpene, squalene, obtained from various plant sources and the livers of deep sea sharks, has utility as a nutraceutical product, is used extensively in many types of cosmetics with claims for its antioxidant properties preventing skin damage, has special utility as a lubricant for high performance machinery, and is used as an adjuvant in many pharmaceutical formulations, enhancing the immune system response and promoting cardiac health (Bhilwade et al., 2010, Huang et al., 2009, Reddy & Couvreur, 2009). Metabolic engineering triterpene production in *Saccharomyces cerevisiae* could provide an alternative supply source for these valuable terpene compounds. The precursors of all classes of terpenes are natively synthesized in yeast via their sterol biosynthesis pathway, which offers unique opportunities to divert these intermediates for the production of high-value terpenes, and triterpenes in particular.

**5.3. Results**

**5.3.1. Metabolic engineering botryococcene biosynthesis in yeast**

Taking advantage of the native mevalonate (MVA) pathway that normally operates in yeast, and introducing a SUE (sterol uptake enhancement) mutation by EMS mutagenesis, and deletion of the squalene synthase gene (*ERG9* locus), our yeast strain ZX178-08 generates a high level of FPP as indicated by the accumulation of its dephosphorylated product farnesol. Since botryococcene would be a novel compound for yeast and not likely catabolized, engineering botryococcene biosynthesis into strain ZX178-08 was considered an important challenge. We hence introduced a chimeric construct fusing 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. The yeast vector pESC-Adh-Ura-SSL1-3M assembled by Niehaus et al. (2011) was transformed into yeast ZX178-08 line to evaluate triterpene production capability for botryococcene as shown in Fig. 5.2. After 10 days, shake flask cultures accumulated 60.37 mg/L of botryococcene and 20.66 mg/L farnesol.

5.3.2. Metabolic engineering squalene production in yeast

5.3.2.1 Knocking out the ERG1 gene

In contrast to botryococcene production in our yeast line, production of squalene would be subject to utilization by the innate sterol biochemical pathway. Hence, we sought to inactivate 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 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 Gal10 promoter, then transformant 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 in manner that it is was toxic to the cells. Based on these two reasons, we sought to delete the squalene epoxidase gene, of ERG1 to enhance the prospects of triterpene engineering.

Our strategies for development of erg1 knockout yeast lines are outlined in Fig. 5.3. Insertional inactivation of the ERG1 gene was created by introducing a fragment containing the G418 resistance gene KanmX4 flanked by 30 bp fragments of the LoxP recombinase system plus everything sandwiched between 400 bp DNA sequences of the 5’ and 3’ ERG1 gene. Introduction of this sequence into ZX178-08 line and subsequent selection yielded many G418 resistant colonies. Yeast having correct homologous recombination between the transformed fragment and desired regions of
ERG1 coding sequence was further confirmed by PCR amplification with primers outside the recombination region as shown in Fig. 5.4 and this series of yeast were named ZXA. The G418 resistance gene Kanmx4 gene cassette can be rescued by loxp and Cre recombination to obtain the ZXC line as shown in Fig. 5.6. A truncated catalytic active HMGR (HMG-CoA reductase), the rate limited enzyme of MVA pathway, was also integrated into ERG1 gene region along with Kanmx4 selection cassette, and the obtained positive yeast line was named ZXB. ZXD line is ZXB line after the Kanmx4 selection cassette was removed by the loxp and Cre recombination reaction. PCR confirmation of insertional knockout of erg1 gene and the successful antibiotic marker rescue of ZXC and ZXD lines are shown in Fig. 5.6.

5.3.2.2. Squalene accumulation in new generated yeast lines ZXA and ZXB

Yeast expression construct with BSS driven by strong constitutive promoter GPD was transformed into ZXA and ZXB lines. Terpene production was quantified by GC-FID analysis and cell growth measured as changes in the OD600 nm. After 12 days of cultivation, ZXA and ZXB control lines accumulated farnesol to 125.26 mg/L with no squalene detected. When the BSS gene was over-expressed in the ZXA line, 17.35 mg/L FOH and 87.18 mg/L squalene accumulated. More encouraging, the ZXB yeast line accumulated 70.24 mg/L FOH and almost 300 mg/L squalene.
**Figure 5.1.** Overall approach for generating yeast lines ZXB and ZXD based on previously generated yeast line ZX178-08 (see Chapter 2).

The newly generated yeast lines have a dispensable sterol biosynthetic pathway because of *erg9* (squalene synthase gene) and *erg1* (squalene epoxidase) knockout mutations, plus introduction of an N-terminus membrane spanning domain truncation, catalytic HMG-CoA reductase gene (*tHMGR*), a rate-limited enzyme of MVA pathway, inserted into the ERG1 gene of strain ZX178-08. The newly generated yeast line should be capable of accumulating high levels of FPP but not metabolizing squalene, and hence would provide opportunities for diverting the FPP from the mevalonate (MVA) pathway for the biosynthesis of desired triterpene compounds.
Figure 5.2. Expression of the *Botryococcus braunii* SSL1-3M gene, a SSL1 and SSL3 fusion plus a membrane-spanning domain at the carboxyl terminal, in the ZX178-08 yeast strain monitored by chemical profiling of the culture’s terpene compounds.

The previously developed yeast ZX178-08 strain, which has a dispensable sterol biosynthetic pathway and a squalene synthase (*erg9*) knock out mutation as illustrated in Fig. 5.1, accumulates high level of FPP that can be diverted to the biosynthesis of triterpenes. A yeast expression vector harboring the *B. braunii* SSL1-3M gene fusion was transformed into ZX178-08 yeast line, and a transformant grown for 10 days in a shake flask culture with periodic chemically profiling by GC-MS for the levels of botryococcene and farnesol accumulation as illustrated in panel B. The terpene levels and cell growth over 10 days in a shake flask culture are shown in panel A.
Figure 5.3. A schematic diagram of the approach to generate yeast strains ZXA-D.

The ZX178-08 yeast strain (described in Chapter 2) was engineered for insertional inactivation of the *ERG1* gene by introducing the G418 resistance gene (KanmX4 flanked by 30 bp of the LoxP sequence (indicated by red triangles)), with a truncated HMGR (tHMGR) gene (ZXB line) or without the tHMGR gene (ZXA line), and with each cassette flanked by 200 bp of DNA sequences for the 5' and 3' region of the *ERG1* gene (not shown) and subsequent selection for resistance to G418. To recover the antibiotic resistance marker (KAnmX4) for future uses, the ZXA and ZXB lines were transformed with a Ura3 vector containing the Cre recombinase gene under the transcriptional control of a galactose inducible promoter, and the obtained transformants cultured in liquid medium with glucose as initial sugar carbon source, and then induced with 2% galactose to express the Cre recombinase and induce the specific LoxP-Cre recombination to release KanmX4 gene. Two days later, the yeast were replicate plated on complete medium with and without G418 to select for lines that had lost the resistance trait. Loss of the KAnmX4 gene from the *erg1* locus was confirmed by follow-up PCR reactions. These latter developed yeast strains were designated as ZXC (without tHMGR insertion) and ZXD (with tHMGR insertion).
Figure 5.4. PCR confirmation for the erg1 knockout mutation.
A) The strategy for insertional knockout of the *erg1* gene in the yeast genome through homologous recombination with a G418 resistance gene cassette is illustrated in upper part of panel A, along with identification of the primers uses to verify the various recombination events (P1 and P2). DNA isolated from 16 independent colonies selected for substitution of the G418 resistance gene for the *ERG1* gene and the genomic DNA from control ZX178-08 line were used as templates to perform PCR reactions with the primers P1 (ERG1-90upF) and P2 (ERG1-100dwR) specific for the genomic DNA surrounding the *ERG1* locus and outside the homologous recombination region. If the KanmX4 gene cassette did insert and replace the *ERG1* gene, the expected amplification product would be 2,596 bp versus 1,681 bp as shown for the wild type ZX178-08 control in the agarose gel picture. All 16 of the G418 resistant colonies gave the expected PCR fragment size of 2,596 bp and were designated as the ZXA series.

B) The strategy of insertional knock out *ERG1* gene in yeast genome through homologous recombination with a G418 resistance gene cassette plus an extra copy of truncated HMGR gene is illustrated in the upper part of panel B, along with identification of the primers uses to verify the various recombination events (P1 – P5). DNA isolated from 5 independent colonies selected for substitution of the G418 resistance gene for the *ERG1* gene and the genomic DNA from the control ZX178-08 were used as templates to perform PCR reactions with primer pair combinations P1-P2, P1-P3, and P4-P5. If the KanmX4 gene cassette plus tHMGR gene did insert and replace the ERG1 gene, the expected amplification product with primers P1 and P2 would be 5,286 bp versus 1,681 bp as shown for the wild type DNA from ZX178-08. Two more primer sets, P1 plus P3 and P4 plus P5, were used to do PCR amplification for further confirming the intact integration. For P1 plus P3 primer set, the expected amplification product is 1,130bp versus no amplification in control ZX178-08. For P4 plus P5 primer set, the expected amplification product is 1,668 bp versus no amplification in control ZX178-08. Colonies 1, 2, 4, and 5 provided all the expected PCR verifications with correct fragment sizes from all three primer set test, and were hence designated as the ZXB series.
Figure 5.5. Terpene chemical profiles for yeast strains ZXA and ZXB over-expressing the *Botryococcus braunii* squalene synthase (BSS) gene.

A yeast expression vector harboring the *B. braunii* squalene synthase gene under the control of the GPD promoter was transformed into the yeast strains ZXA or ZXB to compare terpene accumulation levels. Yeast transformed with the expression vector (in red) are compared to control yeast (in black) in terms of their terpene chemical profiles determined by GC-MS. Aliquots of the cultures were chemically profiled at the indicated times by GC-MS and cell culture growth measured as their change in OD600nm over a 12 day period for the ZXA strain (panel A) and ZXB strain (panel B). The GC chromatograph of BSS expressed in ZXB is also shown in panel C along with its mass spectrum, which is identical to an authentic standard.
**Figure 5.6.** PCR confirmation of the selection marker rescue in new strains ZXC and ZXD lines.
The strategy for rescuing the G418 resistance gene cassette is illustrated in the upper sections of panels A and B. Cre recombinase driven by a galactose inducible promoter on a yeast expression vector was transformed into ZXA and ZXB lines, and transformants selected by plating on SCE-Ura plates. Several single independent colonies were picked and cultivated in SCE-Ura liquid media before inducing expression of the Cre recombinase by 1.8% galactose to the culture. After two more days, the yeast cultures were diluted and plated onto selectable YPDE plates. Forty single independent colonies were then replica plated onto YPDE plates containing G418 to screen for loss of G418 resistance. Six independent clones of each strain (ZXA and ZXB) having lost the G418 resistance ability were cultivated and their genomic DNA isolated for PCR screens using two primers P1 (ERG1-90upF) and P2 (ERG1-100dwR) that are specific for the genomic DNA surrounding the ERG1 locus and outside the homologous recombination region. If the KanmX4 gene cassettes were lost as a consequence of the Cre- LoxP recombination reaction, the expected amplification product would be 825 bp versus 2,596 bp from ZXA, 3,515 bp versus 5,286 bp for ZXB, and 1,681 bp for the ZX178-08 control as shown in agarose gel pictures in panels A and B. A. All 6 colonies for each strain gave the expected PCR fragment sizes, the resulting yeast lines were then screen on plates containing 5FOA evict the Cre plasmid. The resulting yeast lines were designate as ZXC (derived from ZXA) and ZXD (derived from ZXB).
5.4. Discussion

The previously generated yeast line ZX178-08 was further modified and advanced for triterpene engineering with the additional knockout of the ERG1 gene and addition of an extra copy of the catalytically active truncated hamster HMGR (Chappell et al., 1995) integrated into yeast genome. Chappell et al. (1995) previously demonstrated that constitutively expression this truncated hamster HMGR in tobacco led to a 3- to 6-fold increase in the total HMGR enzyme activity and a 3- to 10 -fold increase in the total sterol accumulation. HGMR is a rate-limiting enzyme in sterol biosynthesis, catalyzing the formation of mevalonic acid from HMG-CoA, and is highly regulated by downstream intermediates, which feedback control HMGR degradation (Gardner & Hampton, 1999). Overexpression a truncated version of HMGR by removing a post-transcriptional regulation region at N terminal, only expressing the catalytically active C-terminal region, has been shown to dramatically increase squalene accumulation in yeast (Dai et al., 2012, Polakowski et al., 1998). Confirming those observations again, squalene accumulation level was increased 3.4 times and reaches 297.7mg/L in ZXB line, having one extra copy of tHMGR, and growing the cultures in shake flasks under selection media growth condition.

5.5. Materials and methods

5.5.1. 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. YPD media for growing yeast without selection consisted of 1% Bactoyeast extract, 2% Bacto-peptone, and 2% glucose. YPDE media was YPD media supplemented with ergosterol (40 mg/L) for ergosterol dependent lines. Ergosterol. Ergostrol stocks were 10 mg/mL in 50% Triton X-100, 50% ethanol and kept at -20°C. Minimal media, SCE (pH 5.3), contained 0.67% Bacto-yeast nitrogen base (without amino acids), 2% dextrose, 0.6% succinic acid, 0.14% Sigma yeast dropout solution (-his,-leu,-ura,-trp), uracil (300 mg/L), L-tryptophan (150 mg/L), L-histidine (250 mg/L), L-methionine (200 mg/L), L-leucine (1g/L) and 40
mg/L. Selection media was prepared similarly except without supplementing the media with the indicated reagent based on the yeast auxotrophic makers. All solid media plates were prepared with 2% Bacto-Agar.

5.5.2. Yeast transformation and culture performance

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. About 1 µg of plasmid or about 5 µg of linearized DNA was used per transformation and followed by selection on agar plates of SCE medium lacking specified amino acids for the auxotrophic markers at 30°C. Variable numbers of independent colonies were subsequently picked and used to start 3ml cultures in minimal media for isolating genomic DNA or characterize their terpene production capacities. To verify the correct homologous recombination of knockout ERG1 gene more than 20 independent colonies were picked, cultured and prepared for genomic DNA by using YeaStar™ Genomic DNA Kit (Zymo Research, Orange, CA).

Primers ERG1-90upF and ERG1-100dwR outside the desired homologous recombination region as listed in table 1 were used to screen the positive replacement of ERG1 gene. The PCR amplification fragment from PCR wide type genomic DNA is 1681bp. With positive Adh-Kanmx4-loxp replaced Erg1 yeast genomic DNA as template, the PCR amplification fragment is 2596bp, and the obtained yeast line named ZXA. With Adh-Kanmx4-loxp-tHMGR replaced Erg1 yeast genomic DNA as template, the PCR amplification fragment is 5286 bp, and the obtained yeast line named ZXB. To rescue the selection marker kanmx4, the construct Pesc-URA-Cre, having Cre recombinase driven by gal inducible promoter, was introduced into ZXA and ZXB lines. Positive transformants containing Pesc-URA-Cre were cultivated in SCE-Ura liquid media for 3 days, then the Cre recombinase gene expression were induced by 2% galactose when glucose was depleted from the initial start culture. After two more days, the yeast culture was serial diluted and plated on YPDE plates. Forty independent single colonies from each transformation (ZXA line, and ZXB line) were replica on YPDE and YPDE+G418 plates, such that only the positive lines having lost Kanx4 through loxp-Cre recombination could not grow on plate containing G418. Over 40% of the colonies were positive for loss of the Kanmx4 selection lines, those positive yeast colonies were cultivated and extracted genomic DNA for PCR amplification to confirm the loss of
selection marker with primers ERG1-90upF and ERG1-100dwR. The expected PCR fragments size would change from 2596 bp to 825bp for ZXA line and from 5286bp to 3515bp for ZXB line. Those obtained yeast were re-streaked on minimal medium plus 5-FOA to selectively screen for loss of plasmid pEsc-URA-Cre, resulting in yeast lines designated ZXC and ZXD. The whole procedure of generating ZXA, ZXB, ZXC, ZXD lines are shown in Fig. 5.2.

For terpene production quantification, aliquots of yeast cultures were analyzed by GC-MS after 6 days of incubation at 23°C with shaking. Cultures exhibiting the highest terpene production levels were chosen for further time course studies and archived as glycerol stocks at -80°C. Selected lines were characterized for cell growth and terpene production using 30 mL shake flask cultures. Starter cultures grown to saturation in minimal media were inoculated into 30 ml SCE media and 1 mL aliquots withdrawn at every other day intervals for 10–15 days. Cell growth was monitored as the change in optical density at 600 nm every two days, using appropriate dilutions for cultures at later stages of growth. Terpene production was determined by GC-MS.

5.5.3. GC-MS detection and quantification of terpenes

To determine terpene accumulation levels, aliquots of cultures grown for 6 to 12 days were lysed by acetone and then extracted with hexane and aliquots evaluated by GC-MS. In general, to 1 volume of culture, 1 volume of acetone was added and mixed vigorously and left on bench for 15min to lyse the cells. The sample was then allowed to incubate at room temperature for 10 min before adding 1 volume of hexane containing a known amount of cedrene external standard. The mixture was again mixed vigorously, then centrifuged in a clinical centrifuge for 5 min at maximum speed. The upper organic layer was collected and when necessary, concentrated under a N2 stream to 100 µl. An aliquot of the organic phase (1 µl) was then analyzed by GC-MS with a Varian CP-3800 GC coupled to a Varian Saturn 2200 MS/MS (Varian Medical Systems) using a Supelco SLB-5ms fused silica capillary column (30 m x 0.25 mm x 0.25 µm film thickness. The initial oven temperature was set at 100 °C for 1 min, ramped to 250 °C at 15 °C/min, and then ramped to 300 °C at 5°C/min and held for 1 min more. Relative terpenoid levels were calculated relative to the cedrene external standard.
5.5.4. Construction of the squalene epoxidase synthase ERG1 Knockout mutation

Yeast vector pESC-ADA-URA-SSL1-3M for botryococene production was assembled by Tom Niehaus (Niehaus et al., 2011) and pESC-GPD-LEU-tHMGR was built as described in Chapter 2. The Botryococcus SQS gene was cloned into the NotI and SpeI sites of pESC-GPD-Ura vector, and the resulting construct was named pESC-Gpd-Ura-bSQS in Fig. 5.7. Cre recombinase gene was cloned into NotI and SpeI sites of pESC-URA vector (Stratagene), and the resulting vector was named pESC-URA-Cre in Fig. 5.7.

The primers ERG1F and ERG1R as list in table 5.1 were used to amplify the squalene epoxidase synthase ERG1 gene from the yeast BY4741 genomic DNA by using Takara high fidelity Primestar taq polymerase. The obtained PCR fragment was gel purified, A tailed and ligated into the pGEM-Teasy vector (Promega, WI) by following the manufacture manual. The obtained vector was used as template to run second PCR with primers Erg1-splitF and ERG1-splitR to obtain PCR fragment with deletion of 891bp CDS in the middle and remaining 310bp at 5’ end region and 291bp at 3’ end region of ERG1 gene, which are the target homologous recombination sequences for the ERG1 knock out. After digested with BamHI and self ligation, and transformation to DH5α competent cell, the resulting vector containing left and right arms of homologous recombination region and four restriction enzyme cloning sites in the middle of these two homologous sequences, which can be utilized for sub-cloning selection marker and other genes of interest. The resulting construct was named pGEM-ERG1-split as shown in Fig. 5.8.

The Padh-Kanmx4-Tcyc-LoxP antibiotic selection marker cassette was constructed by assembly from three PCR fragments. Padh promoter was PCR amplified with Padh-loxP-BamHIF and Padh-Kanmx4R primers by using Yep352 vector as template. Kanmx4 selection gene was PCR amplified with Padh-kanmx4F and Tcyc-kanmx4R primers by using PYM-N14 plasmid described by (Janke et al., 2004) as template. Tcyc terminator was PCR amplified with Padh-loxP-BamHIF and Padh-Kanmx4R primers by using the pESC vector as the template. Thus, the resulting three PCR fragments containing homologous regions within each other were gel purified and 250ng of each fragment were mixed together to serve as the template for the secondary assembly PCR reaction to fuse them together to obtain the pAdh-Kanmx4-Tcyc-LoxP cassette, which was then
digested and inserted into pGEM-ERG1-split vector. The resulting construct was used as template to run PCR with ERG1F and ERG1R to obtain PCR fragment for generate ZXA lines. Pgpd-tHMGR-Tadh fragment was amplified from pESC-gpd-LEU-tHMGR vector with primers GPD-BamHIF and Tadh-XhoIIR, and then inserted into the pGEM-ERG1-split vector containing the Kanmx4 cassette. The resulting construct was used as a template to amplify a fragment with the ERG1F and ERG1R primers for building ZXB line, which has a erg1 knock out plus one copy of tHMGR integrated into genome.
Yeast expression vectors were designed for the strong, constitutive expression of the SSL1-3M, bSQS, tHMGR, KanmX4 genes driven by the GPD promoter (Pgpd) or the ADH promoter (Padh) and termination sequences provided by the ADH terminator sequence (T-ADH) or CYC terminator sequence (T-CYC) with auxotrophic marker Ura3 as indicated.
Figure 5.8. A Schematic depiction of the steps used in building the ERG1 knockout vector and the insertional mutation of ERG1 through homologous recombination.

The primers used for construction and screening the homologous recombinants and the successfully rescue of antibiotic selection markers are indicated as colored arrows.
Table 5.1. Primers used for the various molecular manipulations describe in materials

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence</th>
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<tr>
<td>tHMGR-NotIF</td>
<td>GGGGCGGCGCGCAAAACAATGTTGTCACGACTTTTCCGTATG</td>
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<tr>
<td>tHMGR-SpeIR</td>
<td>GACTAGT TCAAGCTGACTTTTCTTGGTCACGTTCTTTG</td>
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<tr>
<td>ERG1F</td>
<td>ATGTCTGCTGTTAAGTTGACCTCAGCTTATTN</td>
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<tr>
<td>ERG1R</td>
<td>TTAACCAATCAACACTCAACAAAC</td>
</tr>
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<td>ERG1-split F</td>
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<tr>
<td>ERG1-split R</td>
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<td>ERG1 90up F</td>
<td>ATCAGAACAATTGTCCAGTTAT</td>
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<td>ERG1100dwR</td>
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<tr>
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<tr>
<td>Cre-NotIF</td>
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</tr>
<tr>
<td>Cre-SpeIR</td>
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</tr>
<tr>
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</tr>
<tr>
<td>Padh-Kanmx4R</td>
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</tr>
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<td>Tadh-XhoIR</td>
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<tr>
<td>Kanmx4R</td>
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Table 5.1 (continued)
Chapter 6: Concluding remarks

The over-arching goal of this thesis was to develop yeast as a production platform for all classes of terpenes, classes of compounds that yeast do not accumulate or synthesize naturally. Our challenge was use the tools of genetics and molecular genetics to create a yeast line that contained a dispensable sterol biosynthetic pathway such that carbon flux down this pathway could be redirected to the synthesis and accumulation of mono-, sesqui-, di- or tri-terpene compounds. For this purpose, we first selected for yeast that could take up and utilized exogenous sterols in place of the ergosterol that the cells normally synthesize to meet their sterol needs. This left the native sterol biosynthetic pathway available for metabolic engineering efforts to redirect carbon flux to desired terpene end products. As illustrated in each of the chapters, specific manipulations were required to affect the biosynthesis of each class of terpenes which included the engineering of novel prenyltransferases and knocking out competing pathways that compete for available intermediates and/or catabolize the final, desired end-products. And while we appreciate that our efforts have been successful in developing production platforms for 100s of mg of terpenes per L of cultural broth, we are equally cognizant that these production levels represent a relative low efficiency of the theoretic maximum based on the percent of total feedstock carbon provided. Hence, we believe there are significant gains in efficiency yet to be realized.

Budding yeast exhibit robust oscillations in oxygen consumption during continuous nutrient-limited conditions (Silverman et al., 2010, Tu & McKnight, 2007, Tu et al., 2007), and half of the genes in the yeast genome are expressed periodically during three oscillatory phases, Ox (oxidative), RB (reductive/building) and RC (reductive/charging) of yeast metabolic cycles (YMC) (Tu et al., 2007). These metabolic cycles drive many changes in intracellular metabolite concentrations. Numerous amino acid precursor, amino acids, nucleotide precursor and TCA (tricarboxylic acid cycle) intermediates increase significantly during the Ox phase (Tu et al., 2007). Acetyl-CoA, the initial precursor for MVA pathway, reaches it peak concentration during the Ox phase. Ideally if the yeast cell could be arrested in the Ox phase, then there might be a chance more acetyl-CoA would be available for terpene biosynthesis.
Yeast cells undergoing the YMC are also synchronized with respect to the cell cycle. Yeast mitotic cell cycle can be divided into the G1 (Gap1), S (synthesis), G2 (Gap2), and M (mitotic) phases as in Fig 6.6 (Fingar & Blenis, 2004). Under nutrient limited conditions, yeast cells typically arrest growth and enter into a quiescent state called G_0 (Cai & Tu, 2012). In G1 phase the cell increases in size until it reaches the G1 checkpoint which promotes Start transition, thus, commitment to S phase and a new round of DNA synthesis and division. TOR (target of rapamycin) kinase cascades affect expression of the key regulatory G1 cyclin molecules, which, in turn, promote execution of START (Fingar & Blenis, 2004). In contrast, rapamycin inhibits cell cycle progression and proliferation by inducing an accumulation of cells in the G1 phase (Fingar & Blenis, 2004). Interestingly, the G1 phase is related to the Ox phase in YMC, in which the concentration of acetyl-CoA reaches its peak. Rapamycin and other drugs known to down regulate sphingolipid synthesis also increase the chronologic lifespan (CLS) in yeast (Huang et al., 2012), which may correspond to another metabolic state conducive to the biosynthesis and production of specialized compounds in yeast. Controlling the metabolic state and arresting yeast to specific cell cycle phases, in which there is more precursor feeding to MVA pathway, could represent another important means to further promote terpene production in yeast.
Figure 6.1. The changes in yeast metabolites occur with respect to cell cycle stage (G0, G1, S, and M) and metabolic cycle phase, OX (growth), RB (division), and RC (survival/quiescence) of budding yeast.

The YMC (yeast metabolic cycle) trace represents absolute dissolved oxygen concentrations in the growth medium. The drop in $dO_2$ indicates oxygen consumption. Target of rapamycin (TOR) integrates with signals from nutrients and growth factors to regulate cell growth and cell cycle progression. Rapamycin or drugs down regulating sphingolipid synthesis increases the yeast chronologic lifespan (Huang et al., 2012). Adapted from (Cai & Tu, 2012, Huang et al., 2012).
Bibliography


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Xun Zhuang, Shuiqin Wu, Joe Chappell, Engineer diterpene production in yeast. *Saccharomyces cerevisiae* (In preparation)

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