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Metabolic Engineering of *Saccharomyces cerevisiae* Towards Increased Production of Terpenes and Characterization of Sterol Biosynthetic Enzymes

by

Elizabeth A. Hart

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

**Doctor of Philosophy**

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January, 2001
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Elizabeth A. Hart

The yeast *Saccharomyces cerevisiae* possesses the endogenous precursor for diterpene production but does not biosynthesize diterpenes. Part I of this thesis describes metabolic engineering of *S. cerevisiae* to achieve diterpene biosynthesis at 500-fold increased production levels (5 mg/L) relative to levels observed in wild type yeast. Induced expression of *S. cerevisiae* genes geranylgeranyl pyrophosphate synthase (BTS1) and a truncated form of 3-hydroxy-3-methylglutaryl CoA reductase (HMG1) in yeast carrying the *upc2-1* allele afforded accumulating geranylgeranyl pyrophosphate, the universal precursor for diterpene biosynthesis. The precursor demonstrated efficient cyclization to 7,13-abietadiene upon coexpression of transformed *Abies grandis* abietadiene synthase in a multiple copy yeast shuttle vector. Similarly, metabolic engineering of *S. cerevisiae* allowed investigation of achieving attenuated sesquiterpene production *in vivo*; those results and possible physiological implications to yeast are discussed. The recombinant strains serve as an alternative means of access to natural products via a novel *in vivo* production system.
Part II describes the characterization of three sterol biosynthetic enzymes. Higher plants catalyze the cyclization of (S)-2,3-oxidosqualene to cycloartenol. A single point mutation demonstrated altered steric contributing to catalytic product specificity; two point mutations are characterized and evaluated. Cycloartenol constitutes a cyclopropyl sterol structure not found in other eukaryotes. In an effort to better understand the roles served by the cyclopropyl sterols, the characterization of the gene responsible for their metabolism is described. Understanding this point of evolutionary divergence can facilitate accurate phylogenetic analysis among eukaryotes.
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ABBREVIATIONS

ADP  adenosine-5'-diphosphate
Ala  alanine
ALA  5-aminolevulinic acid
ATP  adenosine-5'-triphosphate
bp   base pairs
CaCl₂ calcium chloride
Cys  cysteine
DEPC diethylpyrocarbonate
DMF  dimethylformamide
DMAPP dimethylallyl pyrophosphate
DNA  deoxyribonucleic acid
dNTP deoxynucleosides-5'-triphosphates
DTT  dithiothreitol
E. coli Escherichia coli
EDTA ethylenediaminetetraacetic acid
FPP  farnesyl pyrophosphate
GC  gas chromatography
GGPP  geranylgeranyl pyrophosphate
Gln  glutamine
GPP  geranyl pyrophosphate
his  histidine
HPLC  high performance liquid chromatography
IAA  isoamyl alcohol
IPP  isopentenyl pyrophosphate
KCl  potassium chloride
KOAc  potassium acetate
Leu/leu  leucine
Lys  lysine
Met  methionine
MgCl₂  magnesium chloride
MgSO₄  magnesium sulfate
MnCl₂  manganese chloride
MOPS  3-[N-morpholino]propanesulfonic acid
MS  mass spectrometry
MVA  mevalonic acid
NAD(P)H  nicotinamide adenine dinucleotide (phosphate)
(NH₄)₂SO₄  ammonium sulfate
<table>
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<tr>
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<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>Phe</td>
<td>phenalanine</td>
</tr>
<tr>
<td>pA</td>
<td>picoampere</td>
</tr>
<tr>
<td>pI</td>
<td>isoelectric point</td>
</tr>
<tr>
<td>PIPES</td>
<td>1,4-piperazinethanesulfonic acid</td>
</tr>
<tr>
<td>Sc</td>
<td>synthetic complete media</td>
</tr>
<tr>
<td>Ser</td>
<td>serine</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecylsulfate</td>
</tr>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>TMS</td>
<td>tetramethylsilane</td>
</tr>
<tr>
<td>ura</td>
<td>uracil</td>
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CHAPTER 1: INTRODUCTION

Metabolic engineering utilizes recombinant DNA technology to restructure metabolic networks of microorganisms leading to improved production and yields of natural products.\(^1\) This method investigates a synchronous series of transformations, defined as a pathway, to produce metabolites. Metabolic pathway manipulation requires an awareness of inherent complex regulation and a comprehensive understanding of the discrete enzymatic transformations involved. Metabolic engineering recently emerged in response to efforts made towards improving cellular function by modifying and/or introducing specific biochemical processes.\(^2\) This thesis describes metabolic engineering of the sterol biosynthetic pathway; success of similar projects employing this method to sterol biosynthesis are discussed but do not reflect an exhaustive review of this field.

The sterol biosynthetic pathway presents an intriguing focus for metabolic engineering because precursors for the entire class of terpene metabolites are produced \textit{in vivo}. The isoprenoid pathway in yeast constitutes the upstream chemistry in sterol biosynthesis and produces steady-state concentrations of farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP); these compounds serve as precursors for sesquiterpenes and diterpenes, respectively. Therefore, yeast that possesses the ability to actively biosynthesize these precursors can be manipulated to produce higher levels of those same compounds. The remaining factor for biosynthesis of a particular sesquiterpene or diterpene hydrocarbon is heterologous expression of the cyclase.

Heterologous gene expression has proved successful in achieving increased product
yields as illustrated by the recombinant production of carotenoids in both bacteria
(*Escherichia coli*) and fungi (*Candida utilis*). Research has recently flourished to meet
the rising demands for the production of these antioxidants as food supplements;
metabolic engineering has presented the most economically feasible form of mass
production. Two recent reports described recombinant microbes into which a gene
cassette coding for the proteins responsible for catalyzing the three-step conversion of
FPP into lycopene had been inserted; upon expression of the gene cassette, the
recombinant organisms biosynthesized lycopene as evidenced by the red hue of the cells.
However, to increase yields for practical application, further modifications in the fungal
system included manipulations to divert metabolite flow towards lycopene biosynthesis
and away from the metabolic pathway, sterol biosynthesis, providing the precursor for
lycopene. As a result of altering the metabolic flux of sterol biosynthesis, a seven-fold
increase in the biosynthesis of lycopene was achieved.

In contrast, the bacterial system encountered adverse regulatory control effects from
the consumption of carbon to produce lycopene. Therefore, the researchers designed an
artificial regulatory element to mediate dynamic control over the biosynthesis of
lycopene. As a result, the rate of lycopene biosynthesis coordinated to the metabolic
state of the isoprenoid pathway, which provides the precursor for lycopene biosynthesis.
Designated as metabolic control engineering, the approach benefits from quantitating
metabolic flux relative to the activity of a designated enzyme. One can imagine that the
recent availability of DNA microarrays will provide an exciting complement to
investigating metabolic control mechanisms and will aid in the future success of metabolic
control engineering.\textsuperscript{7}

The recombinant biosynthesis of steroids by baker's yeast (\textit{Saccharomyces cerevisiae}) represents a different example describing the utility of metabolic engineering.\textsuperscript{8} A yeast strain normally does not biosynthesize steroids such as pregnenolone and progesterone.\textsuperscript{9} However, yeast does biosynthesize sterols, which serve as the precursors for steroids in mammalian systems. Researchers have successfully exploited the natural biosynthesis of sterols in yeast by inserting heterologous proteins from bovine to process the yeast-specific sterol ergosterol to pregnenolone and progesterone. The recombinant strains emulated the behavior of natural steroid-producing tissues of higher eukaroytes.\textsuperscript{8}

These three examples describe the use of recombinant technology to produce natural products in an unnatural microbial system. Typically, metabolic engineering pursues production of such compounds because the method represents a feasible commercial alternative to synthetic production methods or extraction from natural resources. Moreover, efficient \textit{in vivo} production systems utilize inexpensive and environmentally safe raw materials. Ultimately, the application of metabolic engineering enables the novel biosynthesis of metabolites through the use of existing metabolic pathways. In the following chapters, an example of this application will be described in which unnatural production levels of diterpenes and sesquiterpenes were accomplished by altering the yeast sterol biosynthetic pathway.

Briefly, diterpenes and sesquiterpenes have been isolated in large quantities from plants and marine organisms.\textsuperscript{10} In plants, diterpenes and sesquiterpenes serve as defense toxins, volatile defensive signals, pollinator attractants, and photoprotectants; the role
these compounds serve in marine organisms is speculated to primarily include chemical
defense mechanisms.\textsuperscript{11,12} In addition to the physiological utility imparted to their host,
some sesquiterpenes and diterpenes have exhibited clinical and medicinal relevance.
Unfortunately, limited supplies of many of these potential drugs hampers clinical
investigations, thereby prompting the design of a new production venue. To this end,
metabolic engineering of \textit{S. cerevisiae} has been employed to produce diterpenes and
sesquiterpenes in high yields.

A notable diterpene is taxol, which possess potent antitumor efficacy.\textsuperscript{13} Other
examples can be found in marine organisms, which contain an abundance of diterpenoids
with potential clinical value.\textsuperscript{14} For example, one Caribbean soft coral, \textit{Pseudopterogorgia}
elisabethae, biosynthesizes a family of diterpene glycosides found to induce potent anti-
inflammatory activity in mammals.\textsuperscript{15-17} Currently, the organic extract of \textit{P. elisabethae}
comprises an active ingredient of Estee Lauder sensitive skin products because of this
activity. Due to the heightened commercial demand, further investigations of \textit{P. elisabethae}
revealed six novel diterpenoids which displayed efficacy towards either
tuberculosis\textsuperscript{18-20} or cancer.\textsuperscript{21} Unfortunately, the pseudopterosins are harvested directly
from reefs because the high cost of synthesizing the active isomer(s) prohibits a more
environmentally conscious means of obtaining the compound(s).

Despite the discovery of a myriad of naturally occurring diterpenes, the number of
respective diterpene cyclases that have been characterized is comparatively low.
Functional characterization \textit{in vitro} of diterpene cyclases is frequently impeded by
insolubility of the enzyme leading to low activity.\textsuperscript{12} Some plant diterpene cyclases
include casbene synthase from castor bean,22 kaurene synthases from *A. thaliana*, maize, and pumpkin,23-25 abietadiene synthase from grand fir,26 taxadiene synthase from Pacific yew,26 and levopimaradiene synthase from *Ginkgo biloba*.27 To date no marine diterpene cyclases have been cloned despite the documented clinical usefulness of many such compounds. Presumably, the restricted access to marine organisms combined with the relatively low natural abundance of these diterpenoids hinder biochemical elucidation of diterpene metabolism in marine organisms.

Similarly, marine sesquiterpene cyclases have not been cloned, but a small number of plant sesquiterpene cyclases have been cloned and characterized. Some plants from which sesquiterpene cyclases are available include pepper,28 grand fir,29,30 annual wormwood,31-33 and peppermint.34 Additionally, sesquiterpene cyclases were isolated from the fungi *Fusarium sporotrichioides*35 and *Trichothecium roseum*.36

Production of diterpenes and sesquiterpenes can be tested by incorporation of the respective recombinant cyclase into a microbial system designed to accumulate large amounts of the required substrates (precursors). Engineered microbial systems producing diterpenes or sesquiterpenes would facilitate practical production of the natural products, thereby obviating the need for total synthetic routes or extraction from natural resources to access these classes of natural products. Essentially, a metabolically engineered host functions as a production factory for natural products, and in Part I of this thesis, the design of a novel host for the purpose of biosynthesizing diterpenes and sesquiterpenes is described. Also potential implications to related cellular processes are discussed to guide future strain optimization efforts.
**PART I: BACKGROUND**

**Metabolic engineering of *Saccharomyces cerevisiae* to biosynthesize diterpenes and sesquiterpenes**

This thesis describes experiments conducted to identify mechanisms by which *Saccharomyces cerevisiae* (baker’s yeast) controls the biosynthesis of sterol biosynthetic intermediates with the intent of manipulating sterol metabolism to make large amounts of foreign terpenes *in vivo*. Our investigation focused on two isoprenoid classes: sesquiterpenes and diterpenes. However, the information gained throughout the course of this thesis work indicates that monoterpenes might also be accessible. Although the chemical transformations producing sterols have been studied for many years, the processes that control steady-state concentrations and metabolic flux of *S. cerevisiae* sterol biosynthesis remain an intriguing curiosity. The discussion begins with a brief description of the host organism *S. cerevisiae* and travels down the metabolic pathway to the formation of squalene. This chapter utilizes a convergent approach to introduce and discuss key aspects of previous research in the field of sterol biosynthesis and regulatory mechanisms found relevant during the development of this thesis.

**Saccharomyces cerevisiae**

*S. cerevisiae* served as the microbial host in which *de novo* sterol biosynthesis and corresponding regulatory mechanisms were manipulated. *S. cerevisiae* constitutes a powerful tool to study biochemical aspects of sterol synthesis. The utility of *S.*
*cerevisiae* lies in the fungus’s biochemical flexibility and genetic pliability which has remained unmatched by alternative eukaryotic models as well as the ease of cultivation.\(^{37}\)

Like bacteria, yeast grows on a simple, well-defined medium with a doubling time of 2-4 hours.\(^{38}\) Although unicellular, yeast contains fundamental constituents of higher eukaryotes such as nuclear membranes and cytoplasmic organelles (*e.g.*, mitochondria, endoplasmic reticulum). Indicative of the widespread applications of *S. cerevisiae*, sequence of the entire yeast genome has recently been elucidated,\(^{39}\) thereby facilitating investigations of complete metabolic pathways and regulation networks in a eukaryote. A large amount of research has been reported that describes the biochemical and molecular processes of yeast, but work specifically examining the regulation of yeast sterol metabolism includes only a small subset of these publications. The accurate delineation of regulatory mechanisms controlling sterol biosynthesis will allow access, via bioengineering, to clinically and commercially important secondary metabolites produced by this pathway.

**Sterol Biosynthesis**

This thesis describes work to investigate and ultimately redesign the *de novo* sterol biosynthetic pathway in *S. cerevisiae* to achieve *in vivo* biosynthesis of sterol intermediates at commercially exploitable levels. To understand the sensitivity associated with manipulating basal sterol concentrations, background on sterols and their physiological role follows.
A natural limitation exists in the modification of sterol biosynthesis because of the necessary physiological requirement fulfilled by sterols. Sterols serve comparable and essential physiological roles in higher eukaryotes (Figure 1). Once synthesized, sterols intercalate into the cellular membranes where their hydrophobic presence alters membrane permeability and fluidity, thereby, affecting activity of membrane-bound proteins.\textsuperscript{40} This role is known as a bulk membrane requirement, and a broad range of tetracyclic sterol structures can function to fulfill this requirement.\textsuperscript{41} In contrast, yeast cells are known to utilize strictly ergosterol for an unidentified hormonal process.\textsuperscript{42,43}

Because of the sterol requirement, yeast lacking an intact sterol biosynthetic pathway or grown under conditions that induce sterol-depletion will not remain viable unless sterol is fed to the cells. Incidentally, wild type yeast exclude exogenous sterols when grown in the presence of oxygen.\textsuperscript{44} However, when yeast are cultivated under sterol-depleting conditions, cells will import exogenous sterol from the growth medium; therefore, a natural sterol deficiency occurs when yeast is subjected to an anaerobic growth environment.\textsuperscript{45} Early efforts to understand the physiological effect of oxygen-prohibited growth demonstrated a four-fold decrease in ergosterol content when yeast were grown under semi-anaerobic conditions.\textsuperscript{46} The result suggested that sterol synthesis is controlled fundamentally by a feedback inhibition mechanism in which ergosterol serves as the controlling compound. Because of decreased intracellular sterol, the yeast
lack the ability to form critical cellular components such as cytoplasmic membranes, nuclear membranes, and vacuoles. As a result, the sterol-deprived cells experience a very short life span.

Introducing a genetic mutation in the heme biosynthetic pathway emulates the effect of anaerobic growth. Such a deletion inporphyrin synthesis prohibits epoxidation of the acyclic sterol precursor squalene which leads to an inability to complete the synthesis of ergosterol. Consequently, cells must be supplemented with hemin, unsaturated fatty acid, and methionine in addition to sterol. Until the sterol importation phenotype was observed, a genetic means to isolate sterol auxotrophs was not obvious.
Continued investigation of the sterol uptake mechanism led to the characterization of *S. cerevisiae* SUT1, sterol uptake.\(^{55}\) The *SUT1* gene was cloned by selecting yeast supplemented with ergosterol and transformed with a yeast genomic library for resistance to fenpropimorph,\(^{56}\) a morpholine anti-fungal that inhibits Δ8→Δ7 isomerase (ERG2).\(^{57}\) The resistance was directly linked to the presence of Sut1p, which permitted a five- to twenty-fold increase in uptake of radiolabeled cholesterol.

Another gene, *upc2-1*, implicated in sterol uptake was cloned and characterized ten years after its phenotypic observation.\(^{58,59}\) Cloned by calcium resistance, UPC2 belongs to a superfamily of DNA binding proteins which regulate fungal processes.\(^{60}\) A point mutation in UPC2, incurred upon chemical mutagenesis of wild type yeast and arbitrarily named *upc2-1*, encoded an Asp residue for a Gly residue at amino acid position 888.\(^{59}\) Yeast containing *upc2-1* exhibit aerobic sterol uptake in excess of five-fold relative to wild type.\(^{58}\) More importantly for this thesis, *upc2-1* elicited an increase in the metabolic flux of sterol biosynthesis indicated by the substantial increase in steryl esters detected in yeast carrying the mutant allele. A concomitant decrease in esterification activity was examined as a possible cause for the accumulating acyl esters, but no conclusive evidence was revealed. Therefore, the process by which *upc2-1* induces an overall increase in the rate of sterol biosynthesis remains unknown. Hypersensitivity to Li\(^+\) and Na\(^+\) represent another effect on strains bearing *upc2-1*, which upon exploitation led to the cloning of ENA2, a protein that functions to restore osmotic balance to a cell.\(^{61}\) The sensitivity to monovalent and divalent cations has also been observed in yeast primarily producing sterols other than ergosterol.\(^{62}\)
Although the three mechanisms just described all circumvent sterol absorption, the complete mechanisms controlling sterol uptake remains undiscovered. Despite the incomplete mapping of sterol uptake control, these experiments identified tools that allow cultivation of yeast with the inherent capacity to absorb exogenous sterol. Consequently, a venue for investigating lesions in yeast sterol biosynthesis had been established. The study of yeast blocked in sterol biosynthesis provided invaluable information on regulatory mechanisms controlling sterol metabolism. However, complete inhibition of early enzymes in sterol biosynthesis cannot be isolated because the intermediates produced serve many physiological roles. These chemical transformations constitute a portion of sterol metabolism known as the isoprenoid pathway.

**Isoprenoid Biosynthesis**

Sterol chemistry includes several classes of compounds, such as isoprenoids, carotenoids, sterols, and steroids. Researchers have long been intrigued with the biosynthesis and physiological importance of these compounds. The earliest investigations were undertaken in the late 1880's and involved elucidating the chemical formation of isoprenoids. By 1910, the first Nobel Prize awarded for work in sterol chemistry was given to Otto Wallach of Germany for his work on isoprenoids. Similarly, over fifteen Nobel Prizes would follow that involved pioneering science in the field of sterol chemistry. For purposes of this thesis, the focus will comprise a discussion of two classes of intermediates in the sterol biosynthetic pathway, namely isoprenoids and 4,4-dimethyl sterols.
Yeast sterol biosynthesis requires 27 catalytic steps in the formation of ergosterol, a 4,4-desmethyl sterol (see Appendix I). The catalytic processes leading to the formation of squalene are commonly referred to as the isoprenoid pathway (Figure 2 and 3). The name originates from the isoprene unit (C₅), or isopentenyl pyrophosphate (IPP), which constitutes the building block for isoprenoid biosynthesis, known as "the biogenetic isoprene rule". Easily detected by the integral number of C₅ units in their hydrocarbon skeleton, isoprenoids contribute to critical physiological roles in the cell, including tRNA modification, ubiquinone and dolichol biosynthesis, protein prenylation, and heme A biosynthesis. Like many anabolic pathways, isoprenoid biosynthesis begins with the precursor acetyl-CoA. Restructuring the metabolism of isoprenoids requires first an understanding of the chemical processes leading to IPP formation. Therefore, the following discussion describes the formation of IPP and includes critical experimental observations that aided in the development of this thesis work.

**Biosynthesis of Mevalonic Acid**

Two acetyl-CoA molecules undergo a Claisen condensation to yield acetoacetyl-CoA (Figure 2). Acetoacetyl-CoA thiolase, ERG10, catalyzes this reaction; the enzyme activity responds to cellular sterol content. A third molecule of acetyl-CoA
Figure 2. Biosynthesis of mevalonic acid from acetyl-CoA

condenses at the keto moiety followed by hydrolysis to yield hydroxy-3-methylglutaryl-CoA, HMG-CoA.\textsuperscript{67} Hydroxy-3-methylglutaryl-CoA synthase (HMGS, ERG13) catalyzes this aldol reaction\textsuperscript{68,69} and exhibits similar enzymatic characteristics \textit{in vitro} to ERG10, which will be discussed later in this chapter.

Reduction of HMG-CoA forms mevalonic acid (MVA) and is catalyzed by 3-hydroxy-3-methylglutaryl CoA reductase (HMGR).\textsuperscript{70} The stepwise reduction of HMG-CoA requires 2 moles of NAD(P)H to effectively produce MVA. Results of early experiments indicated that mevaldic acid resulted from the first reduction. The failed attempts to isolate mavallic acid were attributed to a facile second reduction to MVA\textsuperscript{71} due to allosteric binding of the 2 molecules of cofactor necessary for catalysis.\textsuperscript{72} In
mammals, HMGR has been cited as the rate-limiting step in cholesterol biosynthesis.\textsuperscript{73,74} Consequently, the enzyme has become an important target for the clinical development of anti-cholesterimic (cholesterol-lowering) drugs.\textsuperscript{75}

Blocking sterol biosynthesis with selective inhibitors provided a means to clone and characterize several of the yeast sterol biosynthetic enzymes via drug resistance of yeast sterol auxotrophs: HMG1,\textsuperscript{76} HMG2,\textsuperscript{77,78} ERG11,\textsuperscript{79} ERG6,\textsuperscript{80} and SUT1.\textsuperscript{55} Similarly, applications in sterol regulatory studies have proved useful. One such investigation observed the survival of a yeast mutant in heme biosynthesis in the presence of the sterol inhibitor 6-ketocholesterol.\textsuperscript{81} The conditions permitted isolation of cells that acquired the ability to thrive in the presence of the sterol inhibitor. The acquired viability resulted from an increase in HMGR activity leading to an unnatural accumulation of squalene and a consequent decrease in membrane permeability to the sterol inhibitor. Because the inhibitor simulated a lesion in the sterol pathway, sterol metabolism adjusted to non steady-state concentrations to accommodate the stress. In this case, the increase in reductase activity presumably induced a change in membrane permeability by suppressing metabolism of squalene to oxidosqualene. Results from early experiments suggested that HMGR induced increases in the metabolic flux of the entire sterol biosynthetic pathway hence the conclusion that HMGR constituted a rate-determining step of sterol metabolism.

Supporting evidence arose when Lorenz \textit{et al.} recovered yeast mutant in sterol and heme biosyntheses (\textit{hem1 erg7}) with  \deaminolevulinic acid (ALA).\textsuperscript{49} The yeast sterol auxotroph inhibited in sterol uptake exhibited a five-fold increase in HMGR activity.
which directly led to an accumulation of endogenous ergosterol. Further, HEM1 transcription increased in response to eight different sterol inhibitors upon investigation of the genome-wide expression of S. cerevisiae using DNA microarrays. Together these results suggest that intracellular oxygen sensing by heme with sterol biosynthesis as early as the formation of MVA.

Investigating the effect of decreased sterol biosynthesis on enzymatic activity of ERG10, ERG13, and HMGR using temperature-sensitive sterol mutants hinted to a more complex regulatory mechanism. For studies of sterol regulation, temperature-sensitive strains permit gradual changes in steady-state concentrations to be observed as a function of growth temperature. In other words, if the accumulation or the production of a specific intermediate contributes to the regulation of the overall metabolism, then adjusting growth temperatures can facilitate monitoring of this compound. The concentration changes occur because temperature-sensitive mutation(s) permit enzymatic activity of the mutant sterol gene at low growth temperatures. However, because enzymatic activity occurs at relatively low levels, sterol supplementation is required. As growth temperatures increase, the mutant protein begins to denature, thereby creating a lesion in sterol metabolism that exogenous sterol cannot directly recover.

Growing sterol temperature-sensitive sterol mutants under aerobic and anaerobic conditions suggested that the regulation of both Erg10p and Erg13p might be controlled by an unidentified sterol intermediate. Because the enzymatic activity of HMGR remained relatively constant under both growing conditions, excluding yeast mutant in erg 10B and erg13 where only modest activity increases were observed relative to cellular
ergosterol concentration, the researchers contended that mevalonic acid indirectly regulates HMGR activity. This hypothesis extends the previous suggestion that HMGR is the rate-limiting step, to now include the idea that control of HMGR is not solely contingent upon the amount of ergosterol synthesized.

A few months later, Basson et al. identified in S. cerevisiae two isozymes of HMG-CoA reductase, HMG1 and HMG2.\textsuperscript{77} Although yeast containing one functional isozyme remains viable, Hmg1p and Hmg2p exhibit independent regulatory characteristics. Hmg1p is a stable protein transcriptionally stimulated in the presence of oxygen.\textsuperscript{85,86} In contrast, Hmg2p is quickly degraded during aerobiosis under the control of a primary signal derived from FPP and a secondary degradation-enhancing signal preliminarily characterized as an oxysterol.\textsuperscript{87,88} Mammals possess only one reductase enzyme (HMGR), which is regulated by similar chemical signals as HMG2.\textsuperscript{78,86}

Previous attempts to overexpress yeast HMG1 resulted in karmellae formation of the endoplasmic reticulum membrane.\textsuperscript{89} However, removal of the non-catalytic N-terminal transmembrane spanning domain (1656 nucleotides) from Hmg1p permitted the truncated protein to remain in the cytosol rather than the normal membrane location and at least partially nullified the effect of transcriptional regulation on protein integrity.\textsuperscript{90} Examining high-level expression of the truncated Hmg1p under transcriptional control of the ADH1 promoter (alcohol dehydrogenase) yielded an accumulation of squalene 40 times the amount found in wild type. At the same time, intracellular concentrations of late sterol intermediates such as lanosterol and zymosterol increased relative to wild type, but ergosterol concentrations remained constant. The result indicated that yeast HMG1
does not constitute the sole rate-determining step in ergosterol biosynthesis. Co-expression of truncated Hmg1p and Are2p, steryl ester transferase,\textsuperscript{91} showed increases in ergosterol levels in addition to late sterol intermediates.\textsuperscript{92} By increasing the propensity for esterification of accumulating sterols, the researchers designed a cell that could accommodate large increases in the sterol pool by sequestering the excess sterols as acyl esters.\textsuperscript{48} In doing so, the study allowed examination of effects that increased HMG1 activity promotes without triggering feedback inhibition from high-levels of ergosterol. Because increases in ergosterol were observed without adverse physiological effects, the experiment established that increases in the MVA pool corresponded to increases in downstream sterol intermediates.

Promoting increases in sterol intermediates becomes more complicated when the goal includes impeding enzymatic processes. For example, achieving isoprenoid accumulation involves suppressing metabolic flux at points distal to their production. One such target in the development of this thesis has been squalene synthase, ERG9. Recent work indicates that HMGR activity diminishes in the presence of a disrupted squalene synthase (\textit{erg9}).\textsuperscript{93} In contrast, increasing enzymatic activity of ERG20, which catalyzes the formation of FPP, induced only a modest decrease in HMGR activity. This observation presents a model for HMGR regulation that excludes an intimate regulatory connection to FPP production and defines a tight response to decreases in squalene formation. The independent regulation of mevalonate and FPP production will be discussed later in this chapter.
The data compiled and discussed indicate that the reduction of HMG-CoA to MVA contributes to maintaining sterol homeostasis in the cell. The regulatory networks include the presence of a non-sterol intermediate and a direct link to squalene biosynthesis as well.

![Chemical structures and reactions]

Figure 3. Isoprenoid biosynthetic pathway leading to ergosterol

as intracellular ergosterol content. Investigations continue to completely define the regulatory mechanisms controlling HMGR activity. Nevertheless, the rate of MVA
production must influence the subsequent sequence of catalytic events leading to the formation of isopentenyl pyrophosphate (IPP).

**Mevalonic Acid to Isopentenyl Pyrophosphate: The Formation of Isopentenyl Pyrophosphate**

Because the compound provides access to all classes of terpene metabolites, the formation of isopentenyl pyrophosphate (IPP) remains the crux of terpene biosynthesis. The pyrophosphate substituent can act as a leaving group that induces a partial positive charge on the adjacent carbon, thereby permitting electrophilic addition to dimethylallyl pyrophosphate (DMAPP), geranyl pyrophosphate (GPP), or related allylic pyrophosphates (see p. 21). The chemical transformation to form IPP from MVA requires three discrete, enzymatically-catalyzed reactions. First, two separate phosphorylations occur\(^{94-97}\) catalyzed by ERG12\(^{98-100}\) and ERG8,\(^{101}\) respectively; second, the resulting product, mevalonate 5-pyrophosphate, undergoes ERG19-assisted decarboxylation.\(^{102}\) The production of a phosphate ion, isopentenyl pyrophosphate, and ADP accompanies the elimination of carbon dioxide.\(^{84,96}\) Yeast overexpressing ERG19 contains a reduced total sterol content.\(^{103}\) The result suggests that a downstream intermediate or a shunt metabolite functions to feedback inhibit ergosterol biosynthesis. This conclusion had been previously suggested as one mechanism controlling HMGR activity.\(^{66,84}\) Whether the regulating compound is farnesol or geraniol, known feedback inhibitors of ERG12,\(^{98}\) has not been determined.
Once IPP forms, isomerization to the Δ2 isomer, dimethylallyl pyrophosphate (DMAPP), takes place with the aid of isopentenyl pyrophosphate isomerase (IDI1).\textsuperscript{104} The isomerization reaction strongly favors the more thermodynamically stable internal double bond of DMAPP.\textsuperscript{105} Overexpression of IDI1 in \textit{E. coli} equipped with the \textit{Erwinia spp.} carotenoid gene cluster\textsuperscript{106} increased isoprenoid metabolic flux.\textsuperscript{107} The availability of IPP increased, effecting an increase in the subsequent polymerization reactions that utilize IPP as substrate. The increased isoprenoid levels biosynthesized by the recombinant bacterium was inferred from the observed increase in production of the carotenoid β-carotene. Analogous effects have not been observed in yeast overexpressing IPP isomerase.\textsuperscript{107}

The first IPP condensation reaction occurs between one molecule of IPP and one molecule of DMAPP. The resulting dimer contains ten carbons and is known as geranyl pyrophosphate (GPP). Sequential additions of IPP yield the necessary acyclic precursors for all classes of isoprenoids. Only the acyclic precursors FPP and GGPP will be discussed at length in this thesis because experiments have been specifically designed to accumulate these compounds.

**Farnesyl Pyrophosphate**

Farnesyl pyrophosphate synthase, ERG20\textsuperscript{109}, catalyzes the formation of FPP. In yeast, one molecule of DMAPP sequentially condenses with two molecules of IPP to yield all \textit{trans}-FPP.\textsuperscript{110} Yeast utilizes FPP extensively as a precursor not only for ergosterol formation, but also for ubiquinones, dolichols, and protein prenylation. FPP
serves as a major branch-point in the sterol biosynthetic pathway, and as a result strict regulation of its biosynthesis is expected.

Chambon et al. described the isolation of two yeast strains, V134 and V135, which each contained a different ERG20 mutation. Upon cultivation of V134 and V135, V134 excreted geraniol and farnesol into the culture medium. V135 excreted solely farnesol. The results demonstrate that the ERG20 mutations hampered efficient catalysis of the two-step chain elongation reaction producing FPP. The intracellular accumulation of both GPP, the intermediate, and FPP, the product, must have been dephosphorylated prior to excretion into the growth medium. The authors suggested that the removal of the excess prenyl pyrophosphates from the cell occurs to prevent toxic levels of each from being reached. The mechanism for dephosphorylation was attributed primarily to the observed increases in endogenous acid and alkaline phosphatase activities in the mutated strains relative to wild type. However, the hypothesis ignores the reported capacity of mammalian farnesyl pyrophosphate synthase to catalyze the solvolysis of GPP and FPP to the corresponding alcohols. In addition, both mammalian cells and algal cells have demonstrated incorporation of free farnesol into isoprenoid or sterol biosynthesis, respectively, after a presumed in situ phosphorylation.

Because gene disruption of ERG20 leads to cell death, a viable ERG20 deletion seemed unlikely. Indeed, further genetic mapping revealed a second mutation in both yeast strains carrying the allelic mutations in ERG20; the second mutation occurred at squalene synthase, ERG9. The squalene synthase mutation was found to be essential for viability of both V134 and V135. The lack of geraniol excretion in V135 resulted
from a third mutation in ergosterol biosynthesis that specifically mapped to mevalonate kinase, ERG12. Because V135 exhibited a reduced ergosterol content relative to wild type, the third mutation, arbitrarily named \textit{erg}l2-2, seemed responsible for the observed down-regulation of sterol metabolism. The researchers reasoned and confirmed that \textit{erg}l2-2 was necessary for viability of V135 because the mutation decreased geraniol biosynthesis. Considering that geraniol functionally inhibits ERG12, the excretion of the toxic prenyl alcohol by mutating this gene implies that the presence of \textit{erg}l2-2 arose as a spontaneous mutation to increase viability of V135. Did \textit{erg}l2-2 spontaneously alter its secondary structure to negate interaction with geraniol, thereby decreasing sterol metabolism? To understand the physiological effect of \textit{erg}l2-2, the mutation must first be characterized.

Mutations in ERG20 led to prenyl alcohol excretion, which constitutes an unnatural process in yeast. Evaluating the natural response to increased amounts of ERG20 \textit{in vitro} emphasized the importance of strictly controlled FPP synthesis. Such experiments included the examination of wild type yeast overexpressing ERG20 on both a multiple-copy plasmid and an integrative plasmid. The presence of multiple copies of ERG20 elicited a 70 \% increase in ERG20 activity but only a 10 \% increase in ergosterol content relative to wild type yeast. Heterologous expression of the extrachromosomal ERG20 gene increased ERG20 activity only 20 \% with a concomitant 28 \% increase in ergosterol.

The difference in ergosterol content relative to the number of ERG20 copies indicates that, in the former case, the excess FPP triggers a decrease in metabolic flux of sterol biosynthesis suggested by the unchanged levels of ergosterol. The theory finds
strength in the documented inhibition of Erg12p by FPP. These results quantitatively demonstrated the presence of a strict regulatory mechanism associated with high intracellular levels of FPP. This fact poses a problem if, as in the goal of this thesis work, metabolic engineering to accumulate unnaturally high-levels of FPP defines a critical step for successful sesquiterpene production.

To investigate further the physiology of modifying FPP biosynthesis, the erg20-2 mutation was characterized as Lys197Glu. Expression of the yeast strain carrying only this mutation, that is, free of accompanying ERG9 or ERG12 mutations, led to excretion of isopentenol, dimethylallyl alcohol, linalool, and geraniol. The researchers theorized that the Lys residue participates in substrate binding, and the resultant negative charge created by Glu decreases affinity for GPP causing polymerization cessation at C10 rather than at C15.

Extending the scope of altered chain elongation processes, overexpression erg20-2 expressed in a host strain containing erg20-2 and erg9 afforded 100-fold increase in dehydrodolichol biosynthesis. Dolichols require FPP as substrate for cis-prenyltransferase, the first committed step in dolichol biosynthesis, and serve a vital role in protein glycosylation. Upon ERG20 overexpression in yeast possessing an intact sterol biosynthetic pathway, no increases in dolichol biosynthesis were observed. The researchers suggested that dolichols serve as a carbon sink for accumulating FPP in order to maintain undisturbed steady-state concentrations.

A more recent study hypothesized the same fate for accumulating FPP. Upon overexpressing ERG20 in a squalene synthase mutant, erg9, the excess biosynthesized
FPP was incorporated into dolichol biosynthesis; presumably, the incorporation rendered the accumulating farnesol, which possesses cytotoxic activity, innocuous to the cell. In addition, these results suggested that ERG20 retains the capability of trafficking FPP as substrate to either sterol or dolichol biosynthesis. The discovery of SRE-3, a sterol regulatory element binding site in the promoter region of mammalian FPP synthase which induces transcription in sterol-depleted cells, lends validity to the hypothesis that an independent mechanism exists which strictly controls the FPP production.93

Yeast cells place competing demands on FPP which serves not only as precursor for sterol and dolichol biosynthesis, but also for protein prenylation. Because protein prenylation serves a necessary cellular function, impairing this process had to be avoided during the development of this thesis work. The following section briefly describes the chemical basis of protein prenylation with respect to FPP and introduces the second prenylation precursor, geranylgeranyl pyrophosphate (GGPP).

**Protein Prenylation and Geranylgeranyl Pyrophosphate Production**

One type of post-translational modification of proteins occurs upon alkylation of a C-terminal sulfhydryl substituent of a cysteine residue. This process is termed prenylation because prenyl pyrophosphates serve as the alkylating agent. The irreversible formation of a stable thioether confers lipophilic character to a protein, thereby permitting lipid bilayer association; this localization is commonly required for optimum biological activity. Prenylation requires either a C_{15} hydrocarbon unit or a C_{20} hydrocarbon unit. The former takes the form of FPP, and the latter molecule results
from the condensation of one molecule of FPP with one molecule of IPP, yielding a 20-carbon acyclic molecule called geranylgeranyl pyrophosphate (GGPP). Because the biochemical processes controlling GGPP formation proved critical to the success of this thesis, a more thorough discussion is placed at the end of this section.

Yeast contain three prenyltransferases which differ in their recognition motif and their preferred alkylating moiety. Protein farnesyltransferases, PFTases, catalyze the addition of FPP to C-terminal tetrapeptides with the protein sequence CaaX, where “C” is Cys, “a” denotes an aliphatic amino acid, and “X” represents Ser, Met, Ala, Cys, or Gln.121,123 Proteolysis of “aaX” and carboxymethylation of the new terminal Cys immediately follows prenylation to yield an active membrane-bound protein.124 Critical proteins that require farnesylation include fungal mating pheromone a-factor, nuclear lamins, and Ras G-proteins.125,126

Protein geranylgeranyltransferases, PGGTases, use GGPP as substrate and consist of two classes. PGGTases-I alkylate at the same recognition motif as PFTases, but “X” must be Leu or Phe.127 The yeast PGGT-ase I has been recombinantly expressed in E. coli and found to exhibit similar kinetics to yeast PFTase.128 Protein substrates for PGGTase-I include neural G-proteins and Ras-related G-proteins.121 Throughout the last decade, prenylated ras oncoproteins have been an area of intense investigation for cancer treatment.75 The second type of protein geranylgeranyltransferases-II, PGGTase-II, modifies Rab proteins based on the presence of two C-terminal Cys residues in the motif CC and CXC.129 Rab proteins constitute a large family of small GTP-binding
proteins whose physiological contribution to yeast remains unknown; the yeast counterparts are termed Ypt proteins and mediate vesicular traffic in cells.\textsuperscript{130-132} GGPP serves as the prenyl unit for two of the prenyltransferase classes described. Dimeric GGPP synthases, GGPPS, catalyze the formation of GGPP. Those GGPP synthases that fundamentally differ in their compartmental organization efficiently incorporate allylic starter units DMAPP, GPP, and FPP into GGPP synthesis.\textsuperscript{133-136} Organisms that lack the plastid organelle, with the exception of the human brain GGPPS,\textsuperscript{137} have stricter substrate specificity in which relative reaction velocities for DMAPP or GPP do not exceed 15% of reaction velocities for FPP.\textsuperscript{138-141}

To avoid transcriptional and translational complications, the development of this thesis focused exclusively on \emph{S. cerevisiae} GGPPS. The yeast GGPPS, denoted as BTS1, encodes a 335 amino acid protein with a pI = 5.9.\textsuperscript{141} No predictable signal sequence occurs in the 5' flanking region, and in contrast to the human GGPPS,\textsuperscript{142} BTS1 contains no predicative glycosylation sites\textsuperscript{143} but does contain five highly conserved motifs that are present in all known FPP and GGPP synthases.\textsuperscript{144} Upon disruption of BTS1 in wild type yeast, mitotic growth ceases and renders the cells cold sensitive, but vegetative growth remains unperturbed.\textsuperscript{141} Bacterial expression of BTS1 demonstrated strict substrate specificity for FPP as the allylic starter unit. Therefore, efforts to increase production of diterpenes must focus on increasing the pool of FPP available for GGPP synthesis.
Committed Biosynthesis of Sesquiterpenes and Diterpenes

Prenyltransferases constitute one demand for FPP and GGPP. Additionally, each compound serves as a precursor for a sub-class of secondary metabolites known as sesquiterpenes and diterpenes, respectively. The primary focus of this thesis describes steps made towards increasing the \textit{in vivo} production of both sesquiterpenes and diterpenes.

To demonstrate the utility of recombinant yeast for cloning and characterization of diterpene cyclases, this thesis work incorporates heterologous expression of \textit{A. grandis} abietadiene synthase in yeast as a model system. Briefly, partial purification of \textit{A. grandis} abietadiene synthase yielded N-terminal protein sequence permitting PCR amplification of the full-length gene.\textsuperscript{26} Abietadiene synthase, a single polypeptide, demonstrated the ability to catalyze the formation of 7,13-abietadiene from GGPP in a sophisticated multi-step sequence (Figure 4).\textsuperscript{145} Presently, enzymatic control of stereochemistry and enantioselectivity of this complex reaction continues to be investigated.\textsuperscript{146,147}

![Diagram of enzymatic formation of 7,13-abietadiene from geranylgeranyl pyrophosphate](image)

\textbf{Figure 4. Enzymatic formation of 7,13-abietadiene from geranylgeranyl pyrophosphate}
Sesquiterpene cyclases serving as models for this thesis work included trichodiene synthase from *Fusarium sporotrichioides* and epicedrol synthase from *Artemisia annua*. Trichodiene constitutes the committed step towards biosynthesis of a family of mycotoxins called trichotheecenes (Figure 5a). Trichodiene synthase has been cloned and functionally characterized in *E. coli*. In efforts to clone the sesquiterpene precursor that is further metabolized to artemisinin, the antimalarial sesquiterpene, epicedrol synthase from *A. annua* was cloned by two research groups; each used different homology-based methods and further characterized the gene by bacterial expression. *Artemisia annua* produces several detectable sesquiterpenes in variable yields, but epicedrol, a sesquiterpene alcohol (Figure 5b), remained elusive upon organic extraction. Presumably, epicedrol serves as an intermediate metabolite *en route* to the formation of an unknown final product.

![Diagram of enzymatic formation of trichodiene and epicedrol](image)

Figure 5. Enzymatic formation of trichodiene (a) and epicedrol (b)
Identification of intermediates like epicedrol would be facilitated by expression in a recombinant yeast accumulating FPP; one of the goals of this thesis work involves installing machinery to overproduce FPP. However, sequestering FPP for the production of foreign sesquiterpenes leaves reduced amounts available for protein prenylation, ubiquinone and dolichol biosynthesis, and CGPP synthesis. The combined competing demands for FPP are secondary to the requirement by sterol biosynthesis; the pathway consuming the majority of biosynthetic FPP remains sterol biosynthesis.

**Squalene Synthase**

Sterol biosynthesis requires the head-to-head condensation of two molecules of FPP to form squalene, a 30-carbon acyclic unsaturated hydrocarbon. Catalyzed by squalene synthase, ERG9,\textsuperscript{151,152} squalene formation effectively constitutes the first committed step to sterol biosynthesis. This single sub-unit protein contains two catalytic sites, each responsible for one of the reactions described above, and one to four putative transmembrane domains.\textsuperscript{152} The protein associates primarily to the microsomes but remains exposed to the cytosol in order to accept water-soluble FPP as substrate. The chemical transformation occurs in two steps: first, the formation of the highly unstable cyclopropyl intermediate called presqualene diphosphate; second, the cyclopropyl ring is opened followed by NAD(P)H assisted reduction to squalene.

A yeast strain mutant in squalene synthase (*erg9*) hosted overexpression of the *S. cerevisiae* ERG9 on a multiple-copy plasmid which effected a modest two-fold increase in enzymatic activity and an unexplained decrease in ergosterol content relative to wild
type. However, removal of one of the putative transmembrane domains, the hydrophobic C-terminus, allowed protein levels sufficient to allow purification from E. coli. The increase in protein solubility and new cytosolic localization of the truncated protein identified the 25 amino acid C-terminal sequence as the hydrophobic anchor for association to the endoplasmic reticulum. However, the truncated protein partially retained its intrinsic microsomal association when grown in S. cerevisiae (30% of the total ERG9 protein was located in the microsomes) relative to E. coli (> 95% of the total ERG9 protein was located in the microsomes). Kinetic studies indicate that both the full-length and truncated proteins are subject to substrate inhibition, and the rate-determining step in the formation of squalene is its release into the membrane.

Previously, M'Baya et al. showed that squalene synthase specific activity increases between 1.5- to 5-fold when measured in various sterol auxotrophic hosts. The erg10 and erg11 mutant hosts permitted the higher activities, but a mutation at erg12, mevalonate kinase, caused a decrease in squalene synthase activity. This experiment suggested squalene synthase to be partially regulated by the mevalonic acid pool as well as by intracellular ergosterol content. The observed two-fold decrease in ERG9 transcription upon cultivation in semi-anaerobic conditions agrees with observed squalene accumulation upon semi-anaerobic growth, as described previously.

More recently, transcriptional regulation of squalene synthase was studied through expression of an ERG9-lacZ fusion protein. A mutation in HMG1, hmg1, invoked a two-fold increase in ERG9 expression, whereas analysis of a mutation in HMG2, hmg2, resulted in undetectable expression changes. This result coincides with the previously
observed decrease in ergosterol content by 50% in *hmg1* mutant and 20% in *hmg2* mutant.\(^{156}\) Both experiments suggested that *hmg2* cannot solely diminish ergosterol to non-viable levels, but *hmg1* sufficiently decreases the ergosterol levels to a threshold that initiates increases in sterol metabolic flux.

As mentioned, sterols exist in two forms: esters or free alcohols. Yeast ARE enzymes mediate the former process which allows the cell to store excess sterols as an inert acyl ester.\(^{91}\) While ERG9 expression levels are inversely related to ergosterol content, contradictory data have been proposed on the ERG9 transcriptional implications of increased esterification activity.\(^{155,157}\) In the investigation of the ERG9-lacZ fusion, a strain that increased expression six-fold without altering the sterol product profile resulted;\(^{155}\) the strain was named MKY16. The mutation inducing unnaturally high expression of ERG9 could not be linked to an increase in downstream sterol biosynthesis or esterified ergosterol. Consequently, another regulatory element, yet unmapped, contributing to the control of squalene synthesis was postulated. Overall, the investigation concluded that in addition to sterol biosynthetic mutations, transcriptional activation can be correlated to heme activating proteins, *HAP*,\(^{158}\) a heme-induced repressor of hypoxic genes, *ROXI*,\(^{159}\) and the yeast activator protein transcription factor, yAP-1.\(^{160}\)

A *S. cerevisiae* strain capable of sterol uptake and carrying a disrupted *erg9* gene was additionally tested as a host to identify regulatory mechanisms associated with ERG20 and HMGR activity.\(^{93}\) The strain mutated in squalene synthase was cultivated with and without sterol supplementation. Upon sterol supplementation both ERG20 and
HMGR activities decreased relative to wild type. Presumably, the absorbed exogenous sterol meets the sterol requirement such that down-regulation of in vivo sterol metabolism occurs. However, removal of the sterol from the growth medium induced a significant increase in ERG20 activity, a small change in HMGR activity attributed to Hmg2p degradation, and a concomitant decrease in total ergosterol levels relative to the same strain grown with sterol supplementation. Unchanged dolichol levels indicated that the apparent accumulating biosynthetic FPP was not redirected into polyprenol biosynthesis. Consequently, the researchers hypothesize that ERG9 serves the role as enzymatic determinant of the metabolic flux of the isoprenoid pathway.

A more recent study used DNA microarray technology to investigate sterol transcriptional responses to treatment with sterol biosynthetic inhibitors. ERG9 exhibited a transcriptional increase on treatment with an ERG11 inhibitor, fluconazole. Additionally, transcription of the native synthase increased in yeast mutant in erg2 and erg6 (see Appendix I). The observations expand the regulatory mechanisms controlling squalene production to include a response to accumulating 4,4-dimethyl sterols as well as 4,4-desmethyl sterols. In other words, ERG9 activity varies as a result of perturbations in isoprenoid biosynthesis and sterol biosynthesis. With the formation of squalene, the last precursor for isoprenoid biosynthesis is produced. At the same time, one oxidation of squalene commits the pathway to the biosynthesis of sterols suitable for fulfilling the bulk membrane requirement. Therefore, the observed regulation of ERG9 by intermediates both proximal and distal to squalene production seems eminently logical.
In summary, considering the regulation of enzymes participating in FPP and GGPP biosynthesis proved critical throughout the development of this research. The accumulation of these compounds required strategic maneuvering around known and unknown sterol regulatory networks. Consequently, the work described in this chapter significantly contributed to the success of this thesis work.
CHAPTER 2: EXPERIMENTAL PROCEDURES

Materials

Restriction enzymes, DNA polymerase I large (Klenow) fragment, T4 DNA polymerase, calf intestinal alkaline phosphatase, single-stranded binding protein, and M13K07 helper phage were purchased from New England BioLabs. Ligation reactions utilized Fast-Link DNA ligation kit purchased from Epicentre Technologies. Expand high fidelity polymerase kit used for PCR was purchased from Boehringer-Mannheim. Pfu polymerase and pGEM-T vector kit were purchased from Promega. pT7Blue T-vector was purchased from Novagen. Taq polymerase was provided by Prof. B. Bartel (Rice University). Ex-Taq was purchased from Panvera. Zymolyase 100T was purchased from Seikagaku Corporation.

Media ingredients were purchased from Fisher Biotech. Reagent chemicals were purchased from Sigma Chemical Company. Organic solvents were purchased from EM Science. Several GC standards were given as gifts to support the development of this thesis work, and they are as follows: (+)-copalol was a generous gift from Prof. R. Coates; 7,13-abietadiene was a generous gift from H. Schepmann and Prof. R. Croteau; geranyllinalool was a generous gift from Dr. B. Ruan.

Media

Bacteria

All E. coli cultures were cultivated in Luria broth, LB (5 g tryptone, 2.5 g yeast
extract and 2.5 g NaCl dissolved in 500 mL deionized water). The solution was autoclaved for 40 min to sterilize. Solid LB media contained the same ingredients as liquid LB with the addition of 7.5 g agar/500 mL prior to sterilization. *E. coli* selective plates, LB-amp, were impregnated with ampicillin (250 × stock: 25 mg/mL, filter sterilized) to a final concentration of 0.1 mg/mL. Blue-white recombinant *E. coli* selection required, in addition to ampicillin, 5-bromo-4-chloro-3-indoly-β-D-galactoside (X-gal) added to the solid media to a final concentration of 20 μg/mL (200 × stock: 200 mg X-Gal dissolved in 50 mL DMF).

**Yeast**

All yeast were grown at 30°C, unless otherwise specified. Media for yeast contained two sources: a nitrogen source and a carbon source. Nitrogen sources used were nutrient rich, YP (2 × YP: 5 g yeast extract, 10 g peptone dissolved in 500 mL deionized water, then autoclaved 40 min), or synthetic complete (2 × Sc: 1.7 g yeast nitrogen base, 5 g ammonium sulfate, and 2 g amino acid dropout mix dissolved in 500 mL deionized water; using solid NaOH pellets, the solution was brought to pH ~ 6, then autoclaved 40 min). The amino acid dropout mixes used were deficient in leucine, uracil, histidine, or tryptophan; the plasmid marker determined which amino acid mix was used in the preparation of the 2 × Sc.

The carbon sources were sugar solutions of dextrose (2 × D: 20 g dextrose dissolved in 500 mL deionized water, then autoclaved 40 min), or galactose (2 × G: 20 g galactose
dissolved in 500 mL deionized water, then autoclaved 40 min). The latter, galactose, was used to induce heterologous expression of recombinant genes and is, therefore, referred to as inducing media. Liquid media contained equal volumes of a nitrogen source and a carbon source. For solid yeast media, 7.5 g agar/500 mL was added to the carbon sources prior to sterilization. Plates for yeast cultivation contained 15 mL of a nitrogen source, 15 mL of a carbon source containing agar, and appropriate nutritional supplements, if needed. Supplements used were ergosterol (100 × erg: 20 mg ergosterol dissolved in 5 mL ethanol and 5 mL Tween 80), hemin (100 × hem: 13 mg hemin dissolved in 50% ethanol, 5 mM NaOH), cupric sulfate (0.1 M CuSO₄ stock: 638 mg cupric sulfate dissolved in 40 mL deionized water, filter sterilized), cholesterol (100 × chol: 40 mg/mL prepared similarly to ergosterol supplement), nystatin (1 mg/mL dissolved in DMF; diluted with sterile deionized water), and 5-fluoroorotic acid (added directly to selective medium to a final concentration of 1 mg/mL).

Strains constructed during the development of this thesis work that directly impacted increased production of diterpenes or sesquiterpenes are listed in Appendix II.

Methods

E. coli transformations

Chemically competent DH5α E. coli were used for all bacterial propagations. The chemically competent cells were pre-prepared from a fresh colony grown overnight at 37°C on LB agar media. One colony was inoculated into 1 mL LB and grown overnight
at 37 °C. The saturated 1 mL culture was used as inoculum (1 μL) for 500 mL SOC (0.5% yeast extract, 2 % tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, 20 mM glucose) and grown with aeration at 37 °C to A₆₀₀ ~ 0.7. Cells were collected by centrifugation (1500 × g, 15 min, 4 °C). Supernatant was decanted, then each cell pellet was gently resuspended in 80 mL pre-chilled TB (TB buffer: 1.51 g PIPES, 0.83 g CaCl₂, 9.31 g KCl dissolved in 500 mL deionized water; pH was adjusted to 6.7 with NaOH, then 5.44 g MnCl₂ was added, and solution was mixed then filter sterilized) followed immediately with a 10 min incubation on ice. Cells were harvested by centrifugation (1500 × g, 15 min, 4 °C); supernatant was decanted, and cells were gently resuspended in 20 mL ice-cold TB. While gently swirling on ice, 1.5 mL DMSO was added to the cell suspension followed by incubation on ice for 10 min. Cells were dispensed in 200 μL, 300 μL, and 400 μL aliquots to pre-chilled sterile microfuge tubes, immediately frozen in liquid nitrogen, then stored at -80 °C until needed. Titer of this lot of chemically competent cells yielded 1 × 10⁸ transformants/μg plasmid DNA.

For E. coli transformation, one aliquot of chemically competent DH5α cells were ice-thawed then transferred to a sterile microfuge tube containing 10 ng - 1 μg recombinant DNA. The cells were gently mixed with the DNA then incubated on ice for 20 min. The solution was immediately heat-shocked by incubating at 37 °C for 5 min. Next, cells were spread directly on LB-amp or LB-amp-X-Gal plates and incubated overnight at 37 °C. Transformants were inoculated into ~1.5 mL liquid LB-amp and grown overnight with aeration at 37 °C. The cells were cured of the plasmid DNA by alkaline lysis method.
The DNA was analyzed by mapping with restriction enzymes according to manufacturer's instructions.

*E. coli* RZ1032 chemically competent cells were used for site-directed mutagenesis because the strain contains *dut*<sup>−</sup> *ung*<sup>−</sup> mutations that allow incorporation of uracil during replication. These chemically competent cells were pre-prepared as follows. Fresh cells of RZ1032 were grown on LB-agar overnight at 37 °C. One colony was inoculated into 1 mL LB and grown overnight with aeration at 37 °C. The entire volume of culture was used as inoculum for 500 mL LB containing 10mM MgSO₄. The culture was grown to A<sub>600</sub> = 0.68 then transferred to pre-chilled sterile centrifuge bottles for collection of the cells by centrifugation (1500 × g, 15 min, 4 °C). After cells were collected, the supernatant was decanted; cells were resuspended in 100 mL ice-cold TfBI buffer (30 mM KOAc, 50 mM MnCl<sub>2</sub>, 100 mM KCl, 10 mM CaCl<sub>2</sub>, 15 % (v/v) glycerol; filter sterilized) by gently swirling while containers remained on ice. Cells were harvested by centrifugation, resuspended in 10 mL TfBI (10 mM Na-MOPS, pH 7.0, 75 mM CaCl<sub>2</sub>, 10 mM KCl, 15 % (v/v) glycerol; filter sterilized), then immediately transferred to pre-chilled sterile microfuge tubes in aliquots of 400 μL and 500μL. The cells were frozen in liquid nitrogen then stored at -80 °C until needed. Transformation of plasmid DNA followed the same procedure as described for DH5α.

Similarly, for *in vitro* assay of the heterologous protein, *E. coli* BL21(DE3)pLysS (Novagen) was transformed as described for DH5α, but LB-agar selective plates contained carbenicillin (250 × carb stock: 250 mg carbenicillin dissolved in 10 mL...
deionized water followed by filter sterilization) and chloroamphenicol (250 × cm stock: 250 mg chloroamphenicol dissolved in 10 mL methanol; solution was stored at -20 °C).

Gel Purification and DNA Ligation

Gel purification of DNA fragments was performed by preparing a 1 % agarose gel (5 g agarose mixed in 500 mL TAEG buffer; mixture was heated to melt agarose and once cooled to ~55 °C, 5 μL ethidium bromide (10 mg/mL stock) was added, mixed, and poured into Owl EASY-CAST electrophoresis block). Once the gel solidified, enough TAE buffer (50 × TAE stock: 242 g Tris base, 57.1 mL glacial acetic acid, 50 mM EDTA, pH 8.0. 1 × TAEG buffer was prepared by diluting 200 mL 50 × TAE stock in 9.8 L deionized water then adding 2.83 g guanosine followed by intermittent mixing for several hours) was added to completely overlay the gel. The pre-digested plasmid DNA was loaded alongside BstEII-digested λ DNA (250 ng λ DNA (New England Biolabs) digested at 60 °C with BstEII following manufacturer's instruction) to determine fragment size. After separating DNA fragments by electrophoresis, the linear DNA fragment of interest was excised from the gel and purified using Qiaex suspension (Qiagen) according to manufacturer's instructions. Ligation of purified linear DNA fragments utilized Fast-Link DNA ligase (EpiCenter Technologies) according to manufacturer's instructions, and the propagation of the recombinant plasmid resulted from transformation into E.coli.
Yeast transformations

Yeast were cultivated in 5 mL of an appropriate media to an $A_{600} \sim 0.6$. Cells were collected by centrifugation (1300 $\times$ g, 3 min), then the supernatant was decanted. To remove residual media, 500 $\mu$L sterile deionized water was added to resuspend the cell pellet; after cell collection by centrifugation, the pellet was rinsed a second time with 500 $\mu$L sterile deionized water. Following the same procedure for cell collection, 100 $\mu$L sterile deionized water and 50 $\mu$L pre-boiled single-stranded carrier DNA (200 mg Salmon Testes, Type III sodium salt (Sigma Chemical) was dissolved in 100 mL TE8 (10 mM Tris HCl pH 8.0, 1.0 mM EDTA), transferred in 100 $\mu$L aliquots, then stored at -20°C) and 5-10 $\mu$g plasmid DNA were added to the cell pellet. Following mixing, 2 mL yeast transformation buffer (40 % polyethylene glycol 3350, 0.1 M lithium acetate, 1 mM EDTA, 10 mM Tris, pH 7.5, 0.1 M DTT) was added to the cell suspension; after mixing, the reaction incubated at ambient temperature for 8 - 16 hours. Cells were then collected by centrifugation (1300 $\times$ g, 3 min). After the supernatant was decanted, the cells were rinsed once with 500 $\mu$L sterile deionized water then resuspended in 500 $\mu$L sterile deionized water. Aliquots of the transformed yeast were spread on to selective yeast media and incubated at 30°C until colonies were observed, typically 2 - 3 days. Following a second round of selection on the same solid yeast media, the recombinant strain was cultivated in selective liquid media then stored at -80°C as a 1:1 mixture with 85 % (v/v) sterile glycerol.
Polymerase Chain Reactions

Primarily two temperature programs were used. The CAPS program was used with *Taq* polymerase (prepared by B. Bartel lab)\textsuperscript{165} and the PFU program was used with *Pfu* polymerase. The CAPS program was comprised of the following steps repeating through 40 cycles: 95 °C for 30 sec to denature DNA, 56 °C for 30 sec to anneal primer to DNA, 72 °C for 3 min to permit second strand synthesis. The term "hot start" referred to the lengthening of the denaturation step from 30 sec to 4 min; immediately following this step, the program was halted, polymerase was added, then the program was resumed. The PFU program followed a slightly different program: 95 °C for 30 sec, 54 °C for 30 sec, 72 °C for 4 min. All PCR reactions contained 8.5 mM MgCl\textsubscript{2}, 16 mM NH\textsubscript{4}SO\textsubscript{4}, 50 mM Tris HCl, pH 9, 20 pmol forward primer, 20 pmol reverse primer, and 10 nmol dNTPs followed by the addition of deionized water to a final volume of 50 µL, unless otherwise specified. Control reactions, which lacked template, were run in parallel in all reactions.

Amplified DNA was purified by gel electrophoresis and ligated into either T-vector or pBluescript (II) KS\textsuperscript{+} (Stratagene) that had been digested with *EcoRV*. Great American Gene Company or Integrated DNA Technologies, Inc. synthesized oligonucleotides. Texas Health Science Center (Houston, TX) or Lone Star Labs Inc. (Houston, TX) performed sequencing manipulations.

Terpene Accumulation Assay

For small-scale analysis of terpene accumulation, an uninduced culture of the strain
was grown at 30 °C in 5 mL dextrose and synthetic complete media lacking uracil. Those strains bearing two plasmids were grown at 30 °C in 5 mL dextrose and synthetic complete media lacking uracil and tryptophan. The saturated culture was harvested by centrifugation (1500 \times g, 3 min); the cell pellet was rinsed twice with 500 \mu L sterile deionized water to remove residual media. Next, the cells were resuspended in 5 mL sterile deionized water. This suspension was used as inoculum in a 1:1000-fold dilution (5 \mu L) into 5 mL induction media. The induction media contained equal volumes of 2 \times G and 2 \times Sc lacking uracil added to a 25 mL Corex tube pre-prepared with HP-20 diaion resin (~0.35 g wet resin was weighed into a clean Corex tube then overlaid with methanol and allowed to incubate for 15 min; methanol was removed and the resin was rinsed twice with deionized water. The resin was overlaid with 600 \mu L deionized water and autoclaved 15 min). The induced culture was allowed to grow 4 days with aeration at 30 °C; however, the time of growth, growth temperature, and air availability varied between experiments so changes will be specified in the Results section.

Once grown, the cultures are filtered through a Kontes chromatograph column (2.8 \times 25 cm) to remove cellular debris and growth media. The captured resin was rinsed with copious amounts of water then eluted thrice with 2 mL ethanol. Ethanolic eluents were combined and extracted with pentane or hexane. The organic extracts were combined and dried at ambient temperatures under a nitrogen stream.

The large-scale assays were performed as described for the small-scale assay except the filtering apparatus varied. Large glass chromatograph columns (3 \times 40 cm) were used
to filter cells and growth media from the resin. To elute the organic material off of the resin, 300 - 500 mL ethanol was passed over the resin then extracted thrice with pentane. The combined organic extracts were dried over MgSO₄, condensed in vacuo, then stored under nitrogen at -20 °C.

**Analytical Instrumentation and Analytical Methods**

*GC analysis.* GC analysis of terpenes used an HP 6890 series equipped with an Rt-x-5 capillary column (30 m x 0.25 mm i.d., 0.1 μm df), a FID detection system with a split ratio of 1:40 (helium was used as carrier). The oven programs used varied relative to the size of the terpene molecule under observation. Sesquiterpene detection used the following oven program: 70 °C for 1 min, 15°C/min increase to 250 °C and hold for 3 min. Both injector and detector were set to 250 °C. Diterpenes were separated for detection using a temperature program with an initial temperature of 150 °C for 5 min, then 5 °C/min increase to 250 °C and hold for 5 min. Again injector and detector were held at 250 °C. Longifolene (0.25 mg/mL) served as the internal standard for diterpenes. Standards for longifolene, geraniol, farnesol, and geranylgeraniol were purchased from Sigma Chemical Company. Epicedrol standard was purchased from Fluka.

*GC/MS.* GC/MS analysis was performed on VG ZAB-HF GC-MS. The oven programs were those described above for the respective terpene class, and the column used was J&W Scientific DB-5ms (60 m x 0.25 mm i.d., 0.1 μm df). Transfer lines were set to the same temperature as the injector. Ionization by electron-impact was achieved at 70 eV.
and scan time of 4.0 sec.

\(^1H\text{-NMR and }^{13}C\text{-NMR.}\) Nuclear magnetic resonance (NMR) experiments were done on a Bruker AMX500 or Avance spectrometer (500.1 MHz for \(^1H\)) equipped with a 5-mm inverse-geometry broadband probe. \(^1H\) NMR spectra were measured at 25 °C in CDCl\(_3\) solution (generally 5 to 20 mM) and referenced to internal tetramethylsilane. \(^{13}C\) NMR spectra were measured at 25 °C in CDCl\(_3\) solution (5 to 150 mM) and referenced to CDCl\(_3\) at 77.0 ppm. NMR samples (0.5 ml in 507-PP or 528-PP tubes from Wilmad Glass Co.; Buena, NJ) were shimmed with spinning at 16-20 rps to give a narrow (\(W_{1/2} @ 0.5\) Hz), symmetrical line for tetramethylsilane. CDCl\(_3\) (Cambridge Isotope Laboratories; Andover, MA) was 99.8 % D.

\(UV\text{ spectroscopy.}\) The spectrophotometer used for all UV measurements was a Shimadzu UV-1601 that was PC controlled through a RS-232C interface.

**Construction of Recombinant Yeast Strains: Diterpene Production**

**Construction of EHY1**

*Obtaining \(BTS1\) from \(\lambda\) phage.* \(S.\ cerevisiae\) geranylgeranyl synthase DNA (ATCC 70279) was retrieved from \(\lambda\) phage in the following manner. Lyophilized pellet received from ATCC was reconstituted with ~ 1 mL of suspension medium (20 mL 1M Tris HCl pH 7.5 and 5.87 g NaCl in 1 L water was sterilized then autoclaved; 10 mL 1 M sterile
MgSO4 was added after solution cooled.) to entirely dissolve the pellet. This concentrated solution was serially diluted in 100-fold increments. A 100 μL aliquot of the first dilution was added to 0.3 mL of LE392 E. coli cells freshly grown in λhm broth (50 mL sterile LB, 0.5 mL 20% maltose and 0.5 mL 1 M MgSO4). After a 20 min incubation, during which time the phage adsorb onto the bacterial cell wall, 3 mL λ top agarose at 50 °C was added; the entire volume was quickly plated onto a freshly prepared plate containing λ bottom agarose (8 g agarose, 10 g tryptone, 2.5 g NaCl were dissolved in 1 L water then autoclaved; after cooling to ~55 °C, 10 mL 1M MgSO4 was added to the medium). Plates were incubated at 37 °C for 10 h then removed and allowed to cool briefly at room temperature before adding 4 mL of pre-chilled suspension medium and 0.1 mL CHCl₃ directly to top of plate. The plate was stored overnight at 4 °C, thereby, allowing the phage to partition into the liquid medium. The next day the liquid, now containing phage, was removed with a sterile Pasteur pipet and transferred to a sterile 15 mL Falcon tube.

Suspension medium was added to a final volume of 4 mL followed by 800 μL lysis mix (2.5 g SDS, 21.8 g Tris base, 2.42 g Tris HCL, 4.6 g EDTA in 100 mL water) and 100 μL DEPC. After mixing, the sample was incubated in a dry bath at 65 °C for 30 min followed by addition of 1 mL 5 M KOAc (18 g KOAc and 19 g HOAc in 100 mL deionized water) and incubation for 40 min on ice. The white precipitate was collected by centrifugation (1300 × g, 15 min), and the supernatant was transferred to a clean 15 mL Falcon tube containing 4 mL of isopropanol. After mixing, the solution was
dispensed evenly in microfuge tubes and centrifuged for 10 min to pellet the extracted phage DNA. Each DNA pellet was dissolved in 66.5 μL 50 mM Tris HCl, 10 mM EDTA at pH 8.0, 100 μg/mL RNase A then transferred to a common microfuge tube. Sixty-six microliters 5 M NaCl and 52 μL 10 % cetyltrimethylammonium bromide in 0.7 M NaCl were added, and the aqueous mixture was incubated at 65 °C for 10 min. To remove organic materials, the sample was extracted with 400 μL CHCl₃. The aqueous layer was transferred to a clean microfuge tube, mixed with 800 μL ethanol, then incubated at -20 °C for 90 min. The insoluble DNA was collected by centrifugation (14,000 x g, 10 min), washed with 300 μL of 70 % ethanol, then air dried 5 min before resuspension in 500 μL of TE8 (10 mM Tris HCl, 1 mM EDTA at pH 8.0).

Construction of pEH1.0 and pEH1.1. Phage DNA containing S. cerevisiae BTS1 was digested with XhoI and KpnI according to manufacturer's instructions. The 7 kb DNA fragment was purified by gel electrophoresis then ligated into pBluescript (II) KS⁺ digested with the same two enzymes. Propagation in DH5α yielded pEH1.0. To reduce the insert size to 5.7 kb, pEH1.0 was digested with XbaI, and the purified fragment was self-ligated then transformed into DH5α for propagation. The extracted plasmids were tested by restriction mapping to confirm the presence of the S. cerevisiae BTS1 gene; the correct plasmid was named pEH1.1.
Site-directed mutagenesis of pEH1.1. To remove the native promoter of \textit{S. cerevisiae}, a SalI site was installed immediately upstream of the start codon using site-directed mutagenesis. RZ1032\textsuperscript{163} chemically competent cells were transformed with \(\sim 10 \mu g\) pEH1.1. Selection on LB-amp solid media afforded transformants that contained plasmid DNA in which uracil replaces the thymine nucleotides. A single transformant was inoculated into a sterile Falcon tube containing 1 mL 2 \(\times\) YT (10 g yeast extract, 16 g tryptone, 5 g NaCl dissolved in 1 L \(dH_2O\); autoclaved 40 min) and 100 mg/mL ampicillin. The culture was shaken vigorously at 37 °C for 3 hours. After this time, 5 \(\mu L\) M13K07 helper phage was added and the culture was allowed to grow at 37 °C for an additional 1.5 hours. Into a sterile Falcon tube containing 20 mL 2 \(\times\) YT, 100 \(\mu g/mL\) ampicillin, and 50 \(\mu g/mL\) kanamycin, the culture was immediately transferred and allowed to grow at 37 °C for 14 hours. Cells were harvested by centrifugation (8000 \(\times\) g, 10 min, 4 °C); the supernatant was carefully transferred to a clean Nalgene centrifuge bottle. To selectively precipitate the phage particles, 5 mL 20 % PEG-8000 containing 2.5 M NaCl was added, mixed with the phage, then incubated on ice for 1 hour. Cells were collected by centrifugation (8000 \(\times\) g, 10 min, 4 °C) followed by removal of the viscous supernatant. The phage pellet was resuspended in 1.2 mL TE8, incubated on ice for 10 min. Centrifugation allowed collection of residual bacterial cells or cellular debris so the supernatant containing phage was transferred to a clean microfuge tube and re-precipitated with 300 \(\mu L\) 20 % PEG-8000 containing 2.5 M NaCl. After a 15 min incubation on ice, the phage was collected by centrifugation (14,000 \(\times\) g, ambient
temperature). The resulting pellet was fully resuspended in 400 μL sterile water then extracted by adding 400 mL buffer-saturated phenol and 4 mL 5 M NaCl. The bilayer was mixed by vortex then centrifuged to efficiently separate the layers. The aqueous layer was transferred to a clean microfuge tube; a second extraction using 600 μL 1:1 phenol: chloroform and 12 mL 5 M NaCl was performed. To ensure that all volumes of phenol have been removed; 600 μL CHCl₃ was added, mixed, and separated by centrifugation. The aqueous layer was transferred to a clean microfuge tube and 1 mL ethanol was added to precipitate the single-stranded DNA (ssDNA). Following centrifugation for 10 min, the ssDNA pellet was resuspended in 50 μL TE8.

Concentration was determined by UV spectroscopy (1 A₂₆₀ = 36 μg ssDNA/ mL) then adjusted to 0.5 μg/mL.

1 μg of ssDNA of pEH1.1 was incubated with 40 pmol phosphorylated GP5S (Phosphorylation was performed by combining 360 pmol GP5S, 70 mM Tris HCl, pH 7.6, 10 mM MgCl₂, 5 mM dithiothreitol, 1 pmol ATP, 5 U T4 kinase and incubating at 37 °C for 1 hour. Reaction was terminated by addition of 250 pmol EDTA, pH 8.0 followed by incubation at 68 °C for 10 min.) in 1 μL 20 × SSC (20 X SSC: 3 M NaCl, 0.3 M Na₃C₄O₇H₂H₂O, pH 7.0). The reaction was brought to 20 mL with deionized water, then placed at 70 °C and immediately allowed to cool to 33 °C at the rate of ambient cooling. Two controls were performed concurrently which lacked either GP5S or T4 DNA polymerase. The sequence of GP5S is the following 36-mer containing the installed Sall recognition motif (underlined): 5'-TATCTTGCCCTCCATGTCGAC
TCCAGACTCGTAAAC-3'.

Second strand synthesis was performed by adding directly to the previous reaction 10 μL ligase buffer (50 mM Tris HCl, pH 7.5, 10 mM MgCl₂, 10 mM dithiothreitol, 10 mM ATP, 25 μg/mL BSA), 50 pmol dNTPs, 1 U single-stranded binding protein, 1 U T4 DNA polymerase, 1 U T4 DNA ligase. The reaction was incubated on ice for 5 min followed by 5 min at ambient temperature, and finally at 37 °C for 1.5 hr. Reactions were terminated by addition of 200 μL ethanol and 2 μL 5 M NaCl and incubated overnight at -20 °C. Double-stranded DNA (dsDNA) was collected by centrifugation (14, 000 × g, 10 min) followed by resuspension in 10 μL milli-Q water and warmed briefly at 37 °C to ensure dissolution. The success of the incorporation of the mutation was determined by gel electrophoresis upon comparison to starting material and the two control reactions.

The dsDNA containing the SalI site in BTS1 was transformed into DH5α; transformants were analyzed for the presence of the installed SalI site by restriction mapping. The correct plasmid was named pEH1.2 and confirmed by sequencing. Removal of the full coding sequence of BTS1 was performed by double digestion of pEH1.2 with SalI and NotI. The purified insert was ligated into both yeast expression vectors pRS305Gal and pRS426Gal166 that had been digested with the same two enzymes; the plasmids were named pEH1.3 and pEH1.4, respectively. JBY575 (MATα ura3-52 trp1–Δ63 leu2-3,112 his3-Δ200 ade2 Gal+) was transformed with pEH1.3. Transformants were selected on 2 % glucose, 1.5 % agar, and 2 % synthetic complete media lacking leucine by growing at 30°C until colonies were observed (~2-3 days). A
second selection on the same medium yielded EHY1 (MATα pGALI-BTS1::LEU2 ura3-
52 trp1-Δ63 leu2-3,112 his3-Δ200 ade2 Gal$^+$).

*Abies grandis abietadiene synthase.* The entire coding sequence of *A. grandis* abietadiene synthase$^{26}$ was obtained from pGEX22 (generous gift from Prof. R. Croteau) after a sequential digestion using *BamHI* and *NotI*. The purified insert was ligated into pRS426Gal that had been digested with the same two enzymes. Propagation in DH5α followed by purification yielded plasmid DNA of pEH9.0.

*Truncated S. cerevisiae HMG1.* Removal of the N-terminal sequence coding for amino acids 1-552 was achieved by PCR amplification. The *S. cerevisiae* HMG1 gene was amplified using *Pfu* (Promega) using the following synthetic oligonucleotides as primers, which contain restriction sites *SalI* and *NotI*, respectively (underlined): forward, 5'-GTCGACCCATG GACCAATTGGTGAAGAACTGAAGT-3'; reverse, 5'-GCGGCACCTCTTTTTCA AGAGAATACCAATGAC-3'. Forty cycles of the PFU program was used to amplify the ~1 kb DNA fragment from pJR9.2 (generous gift from Dr. J.R. Herrera): 95 °C for 30 sec, 54 °C for 30 sec, 72 °C for 4 min. The amplicon was purified then ligated into prepared pBluescript (II) KS$^+$ (digested with *EcoRV* then dephosphorylated with calf-intestine alkaline phosphatase according to manufacturer's instructions). Following propagation in DH5α, the correct plasmid was named pEH12.0 and sequenced to confirm the integrity of the sequence.

The insert containing the C-terminal coding sequence of HMG1$^{90}$ was removed
from pEH12.0 following digestion with SalI and NotI. After purification, the DNA fragment was ligated into yeast expression vectors pRS305Gal and pRS314Gal (generous gift from Prof. B. Bartel) digested with the same two enzymes creating pEH12.1 and pEH12.2, respectively.

Construction of EHY18

Construction of a-factor Recombinant Yeast Containing Modified BTS1 and HMG1. In EHY1, the LEU2 marker was replaced with a yeast URA3 gene flanked by 1.1kb of direct repeats of bacterial sequence (hisG)\textsuperscript{167}. The 5.0 kb BglII fragment of pNKY85 was transformed into host strain EHY1, and transformants were selected based on a successful recombination of URA3 at the functional LEU2 on the host's chromosome. Initial selection on synthetic complete media lacking uracil afforded all cells that incorporated URA3. However, two sites on the chromosome can accept pNKY85; to confirm that recombination took place at the proper locus, twenty transformants were screened by streaking onto both synthetic complete media lacking uracil and synthetic complete media lacking leucine. Four colonies exhibited survival on the media lacking uracil and concomitant death on the plate lacking leucine; such an observation indicated a non-functional LEU2 gene and, consequently, an appropriate recombination. One of those four colonies, named EHY1.1-6\textsubscript{20}, was inoculated into 5 mL synthetic complete media lacking uracil (2 % glucose) for transformation with BstEII digested pEH12.1. Two rounds of selection on synthetic complete medium lacking leucine and uracil yielded EHY1.3 (MAT\textalpha pGAL1-BTS1::hisGURA3hisG pGAL1-trHMG1::LEU2 ura3-52 trpl-
Δ63 leu2-3,112 his3–Δ200 ade2 Gal⁺), a leucine and uracil prototroph appropriate for the genetic cross required to incorporate upc2-1.

To conserve selective markers, EHY1.3 was cured of the functional URA3 by growing on solid media containing synthetic complete medium, 2 % dextrose, 1 mg/mL 5-flouroorotic acid. The resulting strain was named EHY1.4 (MATα pGAL1-BTS1::hisG pGAL1-trHMG1::LEU2 ura3-52 trp1-Δ63 leu2-3,112 his3–Δ200 ade2 Gal⁺).

Incorporation of upc2-1. To incorporate upc2-1 into EHY1.3, a genetic cross was performed. Haploid strains EHY1.3 (MATα pGAL1-BTS1::hisGURA3hisG pGAL1-trHMG1::LEU2 ura3-52 trp1-Δ63 leu2-3,112 his3–Δ200 ade2 Gal⁺) and SC2-1C (MATα upc2-1 leu2 his3 trp1 ura3) were patched together onto solid medium (YPD, 1.5 % agar) and allowed to grow at 30 °C for 2 days. The patch, containing both diploids and haploids, was streaked onto a diploid selective plate consisting of 2 % dextrose, 1.5 % agar, 400 mM CaCl₂, and synthetic complete medium lacking uracil. Colonies that exhibited a growth advantage relative to the respective haploid strains under these condition were transferred to solid sporulation medium (1 % potassium acetate, 0.05 % dextrose, 0.1 % yeast extract, 5 % agar; autoclaved) and grown at 30 °C. After 4 days, tetrads were dissected onto YPD and allowed to grow at 30 °C for 2 days. Each tetrad that yielded four viable spores was selectivity analyzed for specific genotypes. Each strain was replicated on media, indicated in parentheses, to confirm the chromosomal presence of upc2-1 (YPD containing 400 mM CaCl₂), pGAL1-BTS1 (Sc-uracil), and
pGAL1-trHMG1 (Sc-leucine). Three of the colonies exhibited hypersensitivity to Ca$^{2+}$ and viability without uracil or leucine supplementation. Those positives were transferred to a solid synthetic complete medium containing 5-FOA (1 mg/mL) to cure the functional URA3 gene from the chromosome. After growth for 3 days at 30 °C, two of the strains exhibited the required uracil auxotrophy and were renamed EHY18 (MATa pGAL1-BTS1::his pGAL1-trHMG1::LEU2 ura3-52 trp1-Δ63 leu2-3,112 his3-Δ200 ade2 Gal⁺) and EHY19 (MATa pGAL1-BTS1::hisG pGAL1-trHMG1::LEU2 ura3-52 trp1-Δ63 leu2-3,112 his3-Δ200 ade2 Gal⁺).

To analyze the in vivo diterpene production, all recombinant yeast strains were transformed with pEH9.0. In the case of EHY1, a double transformation with pEH12.2 and pEH9.0 yielded yeast strain EHY1[EH12.2][EH9.0].

**Incorporation of a Squalene Synthase Mutation**

*Genetic cross to incorporate erg9-1.* Yeast containing a temperature sensitive squalene synthase (erg9-1)$^{83}$ was purchased from American Type Culture Collection (ATCC 64031). A small portion of the agar stab was streaked on YPD supplemented with ergosterol (20 mg/L) and allowed to grow at 30 °C until colonies were observed. To ensure the integrity of the strain, 5 plates of YPD and 5 plates of YPD supplemented with ergosterol (20 mg/L) were prepared. The erg9-1 strain was streaked on all 10 plates as well as JBY575, which served as a negative control. One of each type of plate was incubated at various temperatures for 5 d to establish temperature sensitivity: 20 °C, 28 °C, 30 °C, and 37 °C.
After confirming that the *erg9-1* containing strain indeed exhibited poor growth at 37 °C, the strain was mated with EHY1 on solid YPD supplemented with ergosterol (20 mg/L). The plate was incubated at 30 °C for 2 d. To select for diploids, a swab of the mated cells were transferred to synthetic complete media lacking uracil but supplemented with ergosterol (20 mg/L) and grown at 37 °C for 3 d. Those colonies exhibiting a healthier growth rate relative to each parent strain was patched on solid YPD supplemented with ergosterol (20 mg/L) and grown at 30 °C for 2 d. To initiate sporulation, the fresh patch of diploid cells was replicated on solid sporulation media (1 % potassium acetate, 0.05 % dextrose, 1 % yeast extract, 5 % agar; autoclaved) supplemented with ergosterol (20 mg/L). The plate was incubated at 22 °C until tetrad formation was observed (~ 20 d). After which time, a swab of cells was transferred to a sterile microfuge tube; the cell wall was partially digested by addition of 50 μL 1 mg/mL zymolyase in 1 M sorbitol and incubating at ambient for 5 min. The reaction was diluted 10-fold with sterile deionized water. A 10 μL aliquot was applied across the width of a 20 mL YPD plate supplemented with ergosterol (20 mg/L). Tetrads were dissected then allowed to grow at 30 °C until colony formation was observed.

All colonies were selected to determine the chromosomal presence of *erg9-1* (YPDE grown at 37 °C). Those exhibiting the presence of *erg9-1* were selected further for tryptophan auxotrophy and subjected to PCR for amplification of the pGal1-\( BTS1::LEU2\) genetic modification. The genomic DNA was not extracted, rather a small swab of cells were incubated in 0.5 mg/mL zymolyase solution for 30 min at 37 °C. The zymolyase partially digests cell walls by hydrolyzing β1-3 glucose linkages of
laminaripentaose units found in cellular membranes;\textsuperscript{169} the cell digestion reaction (2 μL) served as template in screening for the presence of \textit{pGAL1-BTS1}. A modified CAPS temperature program was used (ZYMO: initial 3 min incubation at 94 °C then Ex-\textit{Taq} polymerase was added; amplification was promoted during 40 cycles of the following program: 94 °C 15 sec, 57 °C 15 sec, 72 °C 1.5 min. Following the last cycle, a 3 min incubation at 72 °C) with the following synthetic oligonucleotides: forward (GalS1)-5'\textendash GCACGCTCCGAACAATA- 3', reverse (BTS1R3)-5'\textendash TGAGGAAGGAGACACCGCT-3'. The forward primer contains internal sequence of the \textit{Gal1} promoter to avoid amplification of the native \textit{BTS1} gene. Controls included amplification of genomic DNA using the above primers as well as a reaction with a forward primer that would amplify the native \textit{BTS1} gene. The positive strain was named EHY36 (\textit{MATα pGAL1-BTS1::LEU2 erg9-1 ura3-52 leu2-3,112 his3–Δ200 ade2 Gal}⁺). A strain lacking the genetically engineered \textit{BTS1} but positive for \textit{erg9-1}, \textit{his3}, \textit{ura3}, and \textit{ade2} was named EHY40 (\textit{MAT erg9-1 ura3-52 leu2-3,112 his3–Δ200 ade2 Gal}⁺) and used as a control strain for \textit{in vivo} production of diterpenes.

**Isolation of 7,13-Abietadiene and Geranylgeraniol**

A 2 L flask containing 1000 mL 2 % galactose, synthetic complete medium lacking uracil, and 5 % (w/v) sterile HP-20 resin was inoculated with 0.1 mL saturated culture of EHY18[EH9.0]. The flask was grown with aeration at 30 °C for 4 days. The saturated culture was filtered to trap the resin, which was rinsed with deionized water to remove any residual cells, cellular debris, or medium. Adsorbed hydrophobic compounds were
eluted thrice with ~150 mL ethanol. The ethanolic eluents were combined and partitioned between pentane and water. The organic extracts were combined, dried over MgSO₄, filtered, then concentrated in vacuo.

Using nearly 2 mg of the total 12 mg organic residue, preparative silver TLC developed thrice with 1:1 (v/v) hexane: ethyl acetate afforded a spot co-eluting with geranylgeraniol standard, Rₜ = 0.59. Silica impregnated with the putative geranylgeraniol was scrapped from plate and eluted with diethyl ether. The ethereal extract was passed over a plug of MgSO₄ then dried under a nitrogen stream. GC, GC/MS and ¹H-NMR analysis confirmed the identity of biosynthesized geranylgeraniol (1.2 mg). The spectroscopic information was compared to synthetic geranylgeraniol (acquired by Dr. B. Ruan).

GC analysis using the diterpene oven program yielded a relative retention time (internal standard = 0.1 mg/mL longifolene) of 4.500 ± 0.100 for geranylgeraniol.

¹H-NMR: δ 1.60 (9H, broad s), 1.68 (6H, broad s), 4.162 (2H, d, J = 6.9 Hz), 5.101 (3H, m), 5.421 (1H, t of septet, J = 7.1 Hz). GC/MS: m/z = 290 (0.2), 272 (4.1), 203 (2.8), 161 (5.6), 147 (5.0), 137 (5.2), 136 (6.3), 135 (10.7), 81 (41.4), 69 (100).

To confirm the identity of abietadiene, 9.6 mg of the same organic extract used to isolate geranylgeraniol was loaded onto a separate preparative TLC plate and developed with 80:20 (v/v) hexane: ethyl acetate. Using a 2 cm (width) score from edge to visualize the components, the entire contents of the plate was removed in six segments; the mobilization corresponded to Rₜ = 0.72, 0.66, 0.26, 0.20, and two large segments of the plate to ensure that all of the material loaded was recovered. Fractions were eluted from
the silica with diethyl ether, filtered through a plug containing MgSO₄, and condensed under a stream of nitrogen. GC, GC/MS, and ¹H-NMR analysis confirmed the identity of the accumulating diterpene to be 7,13-abietadiene based on previously reported spectroscopic information.⁶¹ Synthetic crude standard (a generous gift from H. Schepmann) was purified by similar methodology and, additionally, was used to confirm the structure of the biosynthesized diterpene.

GC analysis using the diterpene oven program afforded a peak co-eluting with authentic 7,13-abietadiene at a relative retention time (internal standard = 0.1 mg/mL longifolene) of 4.000 ± 0.100. ¹H-NMR: 0.788 (3H, s), 0.862 (3H, s), 0.913 (3H, s), 1.005 (3H, d, J = 6.8 Hz), 1.015 (3H, d, J = 6.8 Hz), 5.781 (1H, s), 5.430 (1H, m).

GC/MS: m/z 272 (100), 257 (45), 229 (70), 187 (25), 148 (50), 136 (73), 105 (69), 81 (51), 69 (38).

Ergosterol Content Determination

Induced cultures (50 mL) of JBY575[pEH9.0], EHY1[pEH12.2][pEH9.0], EHY18[pEH9.0], and EHY32[pEH9.0] were grown at 30 °C for 4 days with aeration. Each culture was transferred to a clean 50 mL glass Pyrex tube. Cells were collected by centrifugation (1300 x g, 5 min). Supernatant was decanted, then the cell pellets were washed once with 10 mL 0.1 M potassium phosphate, pH 7.4 buffer containing 1 % (v/v) ethanolic tergitol. Following a second wash with 10 mL 0.1 M potassium phosphate, pH 7.4 buffer, the each cell pellet was resuspended in 1 mL 60 % potassium hydroxide, 1 mL 0.5 % methanolic pyrogallol, and 1.5 mL methanol. Preparation of the reaction reagents
was performed immediately prior to combination with each cell pellet.

The saponification reactions were incubated at 64 °C for 2 h. Each reaction was allowed to cool to ambient temperature then extracted thrice with hexane. The organic extracts were washed twice with 0.1 M sodium phosphate pH 7.4 (~ 3 mL), followed by once with brine (saturated NaCl), and once with deionized H₂O. The resulting organic layers were transferred to a 4-dram vial and condensed under a stream of nitrogen. The residue was resuspended in 100 mL 1:1 pyridine/bis(trimethylsilyl)trifluoroacetamide to effect the formation of the TMS-ether derivative. After mixing, the reaction was allowed to proceed for 1 hour at 40 °C. GC analysis was performed after condensing the silylation reaction to near dryness under a stream of nitrogen then reconstituting the derivatized sterols in toluene. The ergosterol standard was weighed to 0.26 mg and prepared similarly as the samples. The ergosteryl ether standard was diluted to 0.26 mg/mL and 0.13 mg/mL and injected to provide a basis for concentration calculation. Cholestane served as the internal standard for all samples and concentration standards.

**Construction of Recombinant Yeast Strains: Sesquiterpene Production**

**Construction of EHY13**

*Obtaining the Cup1 promoter.* Genomic yeast DNA was extracted from a fresh 10 mL saturated culture of JBY575 (*MATa ura3-52 trp1-Δ63 leu2-3,112 his3-Δ200 ade2* Gal^+). The cells were collected by centrifugation (1300 × g, 5 min). Supernatant was decanted and resuspended in 500 μL sterile deionized H₂O. Cellular suspension was transferred
equally to microfuge tubes and centrifuged (14,000 × g, 5 min) to pellet cells. Supernatant was removed and each pellet was resuspended in 200 μL breaking buffer (10 mM Tris HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 2 % Triton X-100, 1 % SDS). Acid-washed beads and 200 μL 25:24:1 phenol/chloroform/IAA (Ambion) was added to the pellet. After mixing by vortex for 3 min, 200 μL TE8 was added. The samples were centrifuged (14,000 × g, 5 min) to sediment solid particulates.

The supernatant was transferred to a clean microfuge tube and 1 mL cold ethanol was added. The released nucleic acids were sedimented by centrifugation (14,000 × g, 15 min), and the pellet was resuspended in 50 mM Tris HCl, pH 8.0, 10 mM EDTA, 100 μg/mL RNase A to enrich for DNA by digesting RNA into soluble fragments. The digestion was incubated for 5 min at 37 °C. Eighteen microliters 7.5 M NH₄OAc and 1 mL cold ethanol was added to stop the reaction. The samples were stored at -20 °C.

PCR amplification of *S. cerevisiae* Cup1 promoter used 1 mL 1:1 dilution of the prepared genomic yeast DNA as template. The following synthetic oligonucleotides were used as primers and contained restriction sites (underlined) for ease of removal from the T vector: forward-5'-GGTACCGGGCCCGATCCCAATT

CCGACATTGGGCGCT-3', reverse-5'-GTCGACCTCAGGTATGTTATGTG

ATGATTGATTGATTG-3'. The Expand high-fidelity kit (Boehringer Mannheim) was used. In reaction vessel 1, 20 pmol forward primer, 20 pmol reverse primer, 10 nmol dNTPs, and 1 μL template DNA were combined and adjusted to 25 μL with deionized water. Vessel 2 contained 5 μL reaction buffer (20 mM Tris-HCl pH 7.5, 100 mM KCl,
15 mM MgCl₂, 1 mM dithiothreitol, 0.1 mM EDTA, 0.5 % (v/v) Tween 20, 0.5 % (v/v) Nonidet P40, 50 % (v/v) glycerol) and 19.25 µL deionized water. Both vessels were kept on ice until immediately prior to the reaction. To vessel 2, 0.75 µL polymerase was added, mixed, and the entire contents for vessel 2 was promptly added to vessel 1. The CAPS program was immediately started.

The ~500 bp amplicon was purified and ligated into T7Blue T-vector (Novagen). Following propagation in DH5α, the purified plasmid was named pEH3.0. The Cup1 promoter sequence was excised by restriction digest of pEH3.0 with KpnI and XhoI and ligated into yeast expression vectors pRS426 and pRS316 digested with the same two enzymes. The resulting vectors were named pEH3.1 and pEH3.2, respectively. To construct the integrative yeast shuttle vector, pRS305, with a Cup1 promoter, pEH3.0 and pRS305 were digested with ApaI and XhoI. The ligation of these purified DNA fragments afforded pEH3.3.

Obtaining *S. cerevisiae* ERG9. Phage containing the *S. cerevisiae* ERG9 DNA sequence was obtained as a lyophilized pellet (ATCC c9998). A small portion of the pellet was placed on solid LB media impregnated with 100 µg/mL ampicillin and allowed to grow at 37 °C for 12-15 hr. One colony was inoculated into 10 mL LB with 100 µg/mL carbenicillin and allowed to grow 12-15 hr at 37 °C. Cells were harvested by centrifugation (1300 × g, 15 min. Supernatant was decanted, and the cell pellet was thoroughly resuspended in 200 µL buffer (50 mM Tris HCl, pH 8.0, 10 mM EDTA, 100
μg/mL RNase A). An equal volume of lysis buffer (200 mM NaOH, 1% SDS) was added to lysis the bacterial cells and the reaction was allowed to proceed for 5 min. The reaction was quenched by adding 200 μL neutralization buffer (3.0 M potassium acetate pH 5.5) and mixing immediately followed by incubating on ice for ~2 h. Partitioning this aqueous mixture with 600 μL phenol: chloroform removed protein and organic material. The resulting aqueous layer was then extracted with 600 μL chloroform to remove residual phenol. The aqueous layer was transferred to a clean microfuge tube and precipitated by adding 1.2 mL ethanol followed by an overnight incubation at -20 °C. The cosmid DNA was collected by centrifugation (14,000 × g, 10 min); the resulting pellet was rinsed with 500 mL 70% ethanol, then resuspended in 50 μL deionized water.

PCR using the c9998 cosmid DNA as template was performed to obtain 0.9 kb 5' untranslated sequence of S. cerevisiae ERG9. The Expand high fidelity polymerase kit (Boehringer Mannheim) was used as described in the section entitled Obtaining the S. cerevisiae Cup1 promoter except the PFU program was used for amplification of the 5' untranslated region (UTR) of ERG9. Synthetic oligonucleotides were used as primers and consisted of the following sequences containing restriction sites (underlined) for ease of subcloning into expression vectors: forward-5' -GAATTCGCGGCGGCCGCCCAGT AGTAG-3', reverse-5' -GGATCCGCTTCCGATGCAAAGTG-3'. The amplified fragment was purified and ligated into pGEM T-vector (Promega). After propagation in DH5α and restriction mapping, the correct plasmid purified, sequenced and named pEH7.1. The insert 5' UTR fragment of pEH7.1 was excised by restriction digest with
EcoRI and BamHI, purified, and ligated into pEH3.0 digested with the same two enzymes. After propagation in DH5α and restriction mapping, the correct plasmid was purified and named pEH7.2.

PCR amplification of the ERG9 coding sequence (amino acids 1-361) was performed similarly to the amplification of the 5' UTR, but the following synthetic oligonucleotides were used: forward-5'-CTCGAGATGGGAAAGCTATTAC AATTGGC-3'; reverse-5'-AAGCTTGCAGGCGCAGGATCTTCACAGCCAAT TTAGA-3'. The amplified DNA was purified and ligated into pBluescript (II) KS+ (Stratagene) that had been previously digested with EcoRV. Propagation in DH5α and restriction mapping resulted in a correct plasmid; the plasmid was confirmed by sequence to contain the partial coding sequence of ERG9 and was named pEH6.4. Several attempts to amplify the entire coding ERG9 sequence were unsuccessful due to the instability of squalene synthase in E. coli. Such difficulties have been reported previously.153

Both pEH6.4 and pEH7.2 were double digested with Xhol and HindIII. The two purified fragments were ligated together, propagated in DH5α, and purified; the correct plasmid was determined by restriction mapping and named pEH7.3. This plasmid contained 919 bp of ERG9 5' UTR, S. cerevisiae Cup1 promoter, and 1083 bp coding sequence of S. cerevisiae ERG9. To excise the insert for yeast transformation, the plasmid was digested with Not1.

Integration of Cup1p-ERG9. Yeast strain LHY1 (MATα erg9::HIS3 hem1::TRP1 ura3-
52 trp1-Δ63 leu2-3,112 his3-Δ200 ade2 Gal+)\textsuperscript{173} was transformed with NotI digested pEH7.3. Selection for a successful incorporation of the Cup1p-ERG9 DNA (from pEH7.3) into the existing erg9::HIS3 locus was performed by observing growth on three different solid media types: (1) YPD supplemented with hemin (13 µg/mL), (2) SC-histidineD supplemented with hemin (13 µg/mL), (3) YPD supplemented with hemin (13 µg/mL) and cupric sulfate (250 µM). A positive strain exhibited life on condition (1) and condition (3) but death on condition (2); however, slightly smaller colony size was observed on (1) relative to (3). Several positive strains were obtained, and one was named EHY9 (MATa pCUP1-ERG9 hem1::TRP1 ura3-52 trp1-Δ63 leu2-3,112 his3-Δ200 ade2 Gal\textsuperscript{+}).

Construction of EHY10 and EHY13. The lesion in sterol synthesis had been corrected, but the lesion in porphoryin synthesis remained. To remove the existing TRP1 gene from hem1\textsuperscript{+}, EHY9 was transformed with EcoRI digested pLB6.\textsuperscript{166} Selection for a functional HEM1 gene was performed on three different solid media types: (1) YPD supplemented with 250 µM cupric sulfate, (2) SC-tryptophanD supplemented with 250 µM cupric sulfate, (3) YPD. The strains exhibiting life on conditions (1) and (3) but death on condition (2) were selected as positives; one of the positive strains was named EHY10 (MATa pCUP1-ERG9 ura3-52 trp1-Δ63 leu2-3,112 his3-Δ200 ade2 Gal\textsuperscript{+}).

Subsequently, EHY10 was transformed with BstEII digested pEH12.1. Selection was preformed on solid media containing 2 % dextrose, 250 µM cupric sulfate, and
synthetic complete medium lacking leucine. After a second selection on the same solid media, the positive strain was named EHY13 \((MATa \, p\text{CUP1-ERG9} \, p\text{GAL1-trHMG1::LEU2 \, ura3-52 \, trp1-\Delta63 \, his3-\Delta200 \, ade2 \, Gal}^-\)). To analyze the sesquiterpene production in strains containing the pCup1-ERG9 cassette, both EHY10 and EHY13 were transformed with pLH4.12, a multi-copy yeast expression vector containing \textit{A. annua} epicedrol synthase.\textsuperscript{32}

**Incorporation of a Mutation in UPC2**

*Construction of EHY24.* JBY575 was transformed with \textit{BspMI} digested pRX2.1, plasmid containing a 2 μm origin of replication and \textit{S. cerevisiae} farnesyl pyrophosphate synthase (ERG20) under transcriptional control of the Gal1 promoter (a generous gift from R. Xu). Two rounds of selection on synthetic complete media lacking leucine yielded EHY24 \((MATa \, p\text{Gal1-ERG20::LEU2 \, leu2-3,112 \, ura3-52 \, trp1-\Delta63 \, his3-\Delta200 \, ade2 \, Gal}^-\)). For analysis of \textit{in vivo} sesquiterpene production, EHY24 was transformed with pLH4.12. Selection on synthetic complete media lacking uracil afforded EHY24[pLH4.12].

*Strains to Compare \textit{S. cerevisiae} HMG1 to \textit{A. thaliana} HMGR.* Additionally, EHY24 served as the host for comparing HMG-CoA reductase genes from both \textit{S. cerevisiae} (pEH12.2) and \textit{A. thaliana}. The \textit{A. thaliana} HMGR gene\textsuperscript{174} (a generous gift from Prof. G. Fink) was subcloned into yeast expression vector pRS314Gal by K. Krukenberg (Rice University). The resulting plasmid, named p(AthHMG1.3), was transformed into EHY24 concurrently with pLH4.12. Positives were selected on synthetic complete
media lacking tryptophan and uracil. The resulting strains, EHY24[pEH12.2][pLH4.12] and EHY24[pAthHMG1.3][pLH4.12] were analyzed for in vivo sesquiterpene production as described in Terpene Accumulation Assay.

**Incorporation of a Squalene Synthase Mutation**

*Genetic cross to incorporate erg9-1.* To incorporate *erg9-1*, a single colony of the strain was patched with CJ2-A\textsuperscript{59} on solid media supplemented with ergosterol. A second plate was prepared similarly and another single colony was patched with EHY24; both plates were incubated at 30 °C for 2 d to allow for mating. Diploids were selected by growing the mated cells on solid synthetic complete media lacking uracil but supplemented with ergosterol (20 mg/L) at 37 °C. Those colonies exhibiting a healthier growth rate relative to each parent strain was patched on solid YPD supplemented with ergosterol (20 mg/L) and grown at 30 °C for 2 d. To initiate sporulation, the fresh patch of diploid cells was replicated on solid sporulation media supplemented with ergosterol (20 mg/L). The plate was incubated at 24 °C until tetrad formation was observed (~14 d). After this time, a swab of cells was transferred to a sterile microfuge tube; the cell wall was partially digested by addition of 50 μL 1 mg/mL zymolyase in 1 M sorbitol and incubating at ambient for 5 min. The reaction was diluted 10-fold with sterile deionized water. A 10 μL aliquot was applied across the width of a 22 mL YPD plate supplemented with ergosterol (20 mg/L). Tetrad were dissected then allowed to grow at 24 °C until colony formation was observed.

Genetic screening of the resulting strains from crossing with CJ2-A included the
following selective media, which all contained supplemental ergosterol (20 mg/L): (1) YPD growth at 30 °C, (2) YPD growth at 37 °C, (3) Sc-uracil, (4) Sc-leucine, (5) Sc-tryptophan, (6) Sc-histidine, (7) 400 mM CaCl₂. The appropriate strain was named EHY3₁₅. This strain, EHY3₁₅, was transformed with *BstEII* digested pEH12.1; selection on synthetic complete media lacking leucine yielded EHY31 (*MATα pGAL1-trHMG1::LEU2 upc2-1 erg9-1 ura3-52 leu2-3,112 trp1-Δ63 his3–Δ200 ade2 Gal⁺*).

Additionally, EHY3₁₅ was transformed with *BstEII* digested pEH1.3; selection on synthetic complete media lacking leucine yielded EHY32 (*MATα pGAL1-BTSI::LEU2 upc2-1 erg9-1 ura3-52 leu2-3,112 trp1-Δ his3–Δ200 ade2 Gal⁺*).

The resulting strains from crossing with EHY24 were screened on similar condition as (1), (2), and (3). Those strains positive for the presence *erg9-1* were screened by PCR for the presence of chromosomal pGAL1-ERG20 using zymolyase digested cells as template (see ). The ZYMO program was used with the following synthetic oligonucleotides: forward (GalS1) 5'- GCACTGCTCCGAACAATA - 3', reverse (ERG20seqR) -5'-TTTGGAAAGTA TTCACCCAA -3'. The forward primer contains internal sequence of the GAL1 promoter to avoid amplification of the intact native ERG20 gene. The positive strains were named EHY33 (*MATα pGAL1-ERG20::LEU2 upc2-1 erg9-1 ura3-52 leu2-3,112 his3–Δ200 ade2 Gal⁺*), EHY34 (*MATα pGAL1-ERG20::LEU2 upc2-1 erg9-1 ura3-52 leu2-3,112 trp1-Δ63 his3–Δ200 ade2 Gal⁺*), EHY35 (*MATα pGAL1-ERG20::LEU2 upc2-1 erg9-1 ura3-52 leu2-3,112 his3–Δ200 ade2 Gal⁺*), and EHY37 (*MATα erg9-1 ura3-52 ade2 Gal⁺*).
Each strain was transformed with a sesquiterpene cyclase in a multiple copy yeast expression vector: trichodiene synthase (pJR9.2),\textsuperscript{148} and epicedrol synthase (pLH4.12).\textsuperscript{32} Unfortunately, EHY34 was unable to grow in galactose, thereby rendering the strain useless for this study. The trichodiene synthase presented considerable problems in expression and was consequently abandoned in favor of the epicedrol synthase. Therefore, all sesquiterpene accumulation data refer to detectable epicedrol as determined by coelution with authentic standard.
CHAPTER 3: RESULTS AND DISCUSSION

Diterpenes: in vivo Production in Recombinant S. cerevisiae

This chapter analyzes and discusses steps made towards increasing in vivo production of GGPP, the universal precursor for diterpene biosynthesis. Theoretically, increased levels of intracellular GGPP leads to a heightened potential for high-level diterpene production. To realize that objective, a diterpene cyclase was incorporated into recombinant yeast strains. The cyclase permitted formation of a diterpene. The biosynthesis of the diterpene molecule served as a model to establish the utility of metabolic engineered yeast to produce diterpenes in vivo.

Diterpene production in wild type yeast and with increased BTS1 activity

Prior to beginning metabolic engineering efforts, the amount of endogenous GGPP available from wild type yeast that could be diverted towards diterpene production was established. Because cellular demands for GGPP are modest, minimal GGPP available for diterpene production was expected. Wild type yeast JBY575\textsuperscript{175} was transformed with a multiple copy plasmid bearing the \textit{A. grandis} abietadiene synthase, pEH9.0\textsuperscript{145} Approximately 0.01 mg/L (extrapolated from the internal standard longifolene at known concentrations) abietadiene was detected by GC and GC/MS analysis (\textit{m/z} 272). Once biosynthesized, 7,13-abietadiene, the product of abietadiene synthase, was shown to relocate into the induction media. The culture media contains a polyaromatic resin that indiscriminately adsorbs molecules onto its surface. The elution of these adsorbed
molecules from the resin affords the analytical sample. Control analyses included the uninduced culture of JBY575[pEH9.0] and an induced culture of the same strain containing empty plasmid JBY575[pRS426Gα]; neither culture yielded detectable biosynthetic diterpene. The data obtained established that genetic modifications were necessary to produce abietadiene in yields exceeding 0.01 mg/L.

Native yeast provide insufficient GGPP for high-level diterpene production, but the precursors isopentenyl pyrophosphate and dimethylallyl pyrophosphate should be abundantly produced to provide ~1 mg/L ergosterol. GGPP biosynthesis was consequently considered to be the rate-limiting step, and the first modification chosen involved heterologous expression of the *S. cerevisiae* geranylgeranyl diphosphate synthase (BTS1) under transcriptional control of the inducible GAL1 promoter. In theory, the acquired ability to manufacture Bts1p in response to galactose in the growth media leads to increased production of GGPP. The new strain was named EHY1 and transformed with the abietadiene synthase plasmid pEH9.0. Induced cultures of EHY1[pEH9.0] afforded approximately 0.05 - 0.10 mg/L detectable abietadiene. The uninduced culture of EHY1[pEH9.0] exhibited no biosynthetic abietadiene.

**Increasing metabolic flux of the upstream sterol biosynthetic enzymes by manipulating HMG1**

Increasing the amount of a rate-limiting intracellular enzyme by introducing an exogenous gene, exemplified in EHY1, constitutes one approach commonly employed in metabolic engineering. However, as in the case of EHY1, additional mutations are often
necessary for high-level production. The approach pursued required increasing metabolic flux through the sterol biosynthetic pathway to increase the biosynthetic rate of the diterpene precursor GGPP. The HMG-CoA reductase (HMGR) enzyme posed an attractive site to enhance the metabolic flux primarily because the protein displays rate-limiting characteristics in sterol biosynthesis. We reasoned that overexpressing HMG1 might effect a concomitant increase of carbon into sterol biosynthesis and subsequently GGPP biosynthesis. However, previously described attempts to overexpress HMG1, one of the two isozymes responsible for the coordinated reduction of HMG-CoA to MVA in yeast, resulted in karmellae formation of the endoplasmic reticulum membrane and, more importantly, an unaltered sterol biosynthetic pathway. On the other hand, overexpression of an HMG1 that lacked the N-terminal spanning domain resulted in an accumulation of squalene 40 times the amount found in wild type yeast but did not affect sterol levels. The soluble truncated Hmg1p increased metabolic flux of the sterol pathway up to the biosynthesis of squalene, but squalene synthase then became rate-limiting. Incidentally, increasing the copy number of the recombinant plasmid did not increase squalene accumulation.

The increased production of squalene implies that its precursor FPP was also overproduced. Because FPP is also a precursor for GGPP biosynthesis (Figure 3), the observed enhancement in metabolic flux to FPP might facilitate GGPP overproduction. Therefore, the S. cerevisiae HMG1 was obtained by a PCR strategy to include coding sequence for amino acids 545-1054 as well as the installation of a start codon (ATG) in-frame with the first coding residue. The plasmids constructed in yeast shuttle vectors
pRS305Gal and pRS314Gal were named pEH12.1 and pEH12.2, respectively. The plasmids differed in their selectable markers (LEU2 and TRP1, respectively) as well as the type of yeast expression vector (integrative and low-copy, respectively). Both pEH12.1 and pEH12.2 contain the GAL1 inducible promoter by which transcription of truncated HMG1 is controlled. To apply the observed increase in sterol metabolism through overexpression of a truncated HMG1, EHY1 was cotransformed with pEH12.2 and pEH9.0. The resulting strain, EHY1[pEH12.2][pEH9.0], yielded approximately 1.0 mg/L extrapolated from the longifolene internal standard injected at a known concentration. Curiously, varying geranylgeraniol (GGOH) levels were detected following induction of EHY1[pEH12.2][pEH9.0].

A likely explanation for the accumulating GGOH in EHY1[pEH12.2][pEH9.0] identifies that abietadiene synthase has become rate-limiting which clearly accounts for the accumulation of diterpene precursor, GGPP. This excess GGPP could easily be hydrolyzed in the cytoplasm to produce intracellular GGOH, a known cytotoxin,\textsuperscript{177} which is then excreted from the cell. Also conceivable is that the increased biosynthesis of GGPP stimulates a regulatory cascade that facilitates its hydrolysis then export into the medium. This latter proposal does not necessarily require that the cyclization step had become rate-determining in diterpene production, but rather that the regulatory network associated with controlling GGPP biosynthesis had been triggered. Another possibility includes the low levels of endogenous GAL4-encoded protein, which has been demonstrated to become rate-limiting when cells contain multiple copies of an
induced GAL promoter. The latter mechanism may be compensated by a concomitant overexpression of the GAL4 protein.

Should these models that speculate an active transport system accurately depict the detection of GGOH in the culture media, an intriguing question is raised; by what transport mechanism effects removal of excess GGPP or GGOH from the cell? The same question can be asked for the excretion of abietadiene. Simple diffusion has been suggested for the previously reported detection of geraniol and farnesol in the culture media of a yeast mutant in farnesyl pyrophosphate synthase (ERG20). Simple (passive) diffusion through the lipid bilayer could account for the mechanism by which abietadiene and GGOH are excreted from the cell. If the mechanism is passive, then the compounds would diffuse based on a concentration gradient until an equilibrium is reached implying that significant amounts of abietadiene and GGOH are left in the cell. However if GGOH is toxic, then an affect on culture saturation levels should be observed. Because of the many questions that arose during the investigation of EHY1[pEH12.2][pEH9.0], further experiments were designed to characterize the excretion of GGOH while simultaneously continuing efforts to improve in vivo diterpene production.

Effect of diterpene cyclization on detectable amounts of geranylgeraniol

Previous studies indicated that accumulation of prenyl alcohols in vivo is toxic to S. cerevisiae. Similarly accumulation of GGPP, which is hydrolyzed by an uncharacterized mechanism to the prenyl alcohol GGOH, may adversely effect doubling
time and/or maximum saturation levels of induced cultures resulting in lower optical densities. Reasoning that cell densities may increase if the steady-state concentration of GGPP is lowered by cyclization to abietadiene, optical densities of induced cultures expressing abietadiene synthase were compared to optical densities of induced cultures lacking the cyclase activity.

To compare growth rates and saturation levels, one induced culture of each strain was processed for excreted organic compounds following daily spectroscopic measurements ($\lambda = 600$ nm). In EHY1[pEH12.2][pEH9.0] the optical density values increased insignificantly after Day 3 indicating that the strain had reached stationary phase.

<table>
<thead>
<tr>
<th></th>
<th>EHY1[pEH12.2]</th>
<th>EHY1[pEH12.2][pEH9.0]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 2</td>
<td>$8.52 \pm 0.54$</td>
<td>$9.30 \pm 0.24$</td>
</tr>
<tr>
<td>Day 3</td>
<td>$9.72 \pm 0.60$</td>
<td>$13.04 \pm 0.38$</td>
</tr>
<tr>
<td>Day 4</td>
<td>$8.78 \pm 0.78$</td>
<td>$13.42 \pm 0.70$</td>
</tr>
<tr>
<td>Day 5</td>
<td>$14.32 \pm 0.12$</td>
<td>$14.08 \pm 0.12$</td>
</tr>
</tbody>
</table>

Absorbance taken at $\lambda = 600$ nm in a 20-fold dilution. Designation of growth time corresponds to the day the respective cultures were processed.

within the time interval of the experiment (Table 1). In contrast, EHY1[pEH12.2] showed significant growth only between Days 4 and 5. Comparing the two strains after 48 h of growth (Day 2) indicates no difference in optical density measurements. This result can probably be attributed to the lag phase that occurs when using galactose as the sole carbon source. Higher optical densities are achieved as growth time is extended.
By Day 3, optical densities of EHY1[pEH12.2][pEH9.0] cultures slightly increased relative to the EHY1[EH12.2] cultures. This difference continues until Day 5 in which EHY1[pEH12.2] exhibited a near doubling in optical density. Assuming that this dramatic increase does not reflect the presence of a spontaneous mutation, the data suggest that the cyclase activity in EHY1[pEH12.2][pEH9.0] does not contribute to a detectable growth advantage.

After optical density measurements were taken, the induced cultures were processed for excreted GGOH and abietadiene (Figure 6). The peak areas obtained by GC analysis are reported in picoamps (pA). The unit refers to the abundance in each injected sample as measured electronically by the flame-ionization detector. The Day 2 induced culture of EHY1[pEH12.2][pEH9.0] produced undetectable levels of GGOH. In general, GGOH production accelerated greatly between Days 2 and 3 then more slowly between Days 3 and 5. Growing beyond Day 4 afforded no increases in GGOH suggesting that GGPP biosynthesis (measured indirectly as the excreted alcohol derivative) remains contingent on the metabolic flux of sterol biosynthesis. Because sterols serve as bulk membrane components, which are not required in large quantities for vegetative growth,41 sterol biosynthesis probably slows in conjunction with mitotic growth.

Assuming that GGOH levels directly reflect the amount of GGPP biosynthesized by the recombinant strains, EHY1[pEH12.2] on Days 4 and 5 biosynthesized 40 - 60 % more GGPP than EHY1[pEH12.2][pEH9.0] on Days 4 and 5. The difference can be explained by the detection levels of abietadiene would account for the percent difference
between the strains. Theoretically, the cyclization to produce abietadiene consumes part of the entire GGPP pool; therefore, the sum of carbon found in the form of GGOH and abietadiene should nearly equal the total amount of GGOH detected in the same strain lacking cyclase activity. The solid line in Figure 6 depicts the data compiled to test the application of this concept.

![Graph](image)

**Figure 6.** Comparison of detectable geranylgeraniol and abietadiene in induced cultures: EHY1[pEH12.2] and EHY1[pEH12.2][pEH9.0]
The amount of GGOH detected in EHY1[pEH12.2] (dotted line) clearly exceeds the sum of GGOH and abietadiene detected upon concomitant overexpression of pEH9.0 (solid line). Moreover, the amount of GGOH detected in EHY1[pEH12.2][pEH9.0] (broken line) is considerably less than the analogous strain lacking cyclase activity. Assuming that the amount of detectable GGOH accurately reflects the amount of biosynthesized GGPP, the data suggest that unequal amounts of GGPP are biosynthesized by the two recombinant strains. Possible explanations for the observed imbalance include the initiation of a regulatory cascade(s) and the ability of geranylgeranyl pyrophosphate synthase (BTS1) to use an additional carbon source for GGPP biosynthesis. Because strict regulation exists to maintain appropriate sterol levels, any disturbance of those intracellular levels could trigger a mechanism responsible for maintaining homeostasis with regards to the rate FPP is diverted to GGPP, the intracellular GGPP levels, or the intracellular GGOH levels. Although no efforts have yet been made to abolish enzymatic processes competing for GGPP, its accumulation represents an unnatural occurrence in yeast cellular metabolism. A reasonable cellular response would then be to counteract the perturbation.

The second possibility assumes that the sum of the amount of detectable abietadiene and GGOH accurately reflect the amount of carbon available for GGPP biosynthesis. Because mammalian tissues have demonstrated a stepwise addition of IPP to DMAPP, GPP and FPP en route to GGPP, precedent exists that Bts1p could accept substrates other than FPP and IPP. However, Jiang et al. reported that BTS1 (expressed in \textit{E. coli}) produced no enzymatic product in the absence of FPP.\textsuperscript{141} Based on the
information observed and previously reported, the increase in GGOH detected in EHY1[pEH12.2] cannot be definitively explained at this point in the project. Even so, one solid conclusion can be drawn; the in vivo diterpene production system had not reached a level for commercial utility.

**Incorporation of upc2-1**

The next step towards increasing diterpene production involved another potential boost in the metabolic flux of sterol biosynthesis. Lewis et al. described a *S. cerevisiae* strain that displayed a pleiotropic mutation in sterol metabolism.\(^{58}\) The strain was characterized as defective in sterol uptake control (*upc2-1*) because of its ability to accumulate exogenous sterol despite an intact and fully functional sterol biosynthetic pathway. Additionally, the *upc2-1* strain displayed an unexplained increase in the metabolic flux of sterol biosynthesis. The latter observation indicated that *upc2-1* may be useful for purposes of increasing production of GGPP without modifying sterol biosynthetic enzymes, which avoids the risk of initiating a feedback inhibition of sterol biosynthesis.

The ability of yeast containing the *upc2-1* allele to import exogenous sterol could be an asset if deletions in the native sterol pathway became necessary to further increase yields of diterpene production. Earlier in this project, efforts to incorporate this importation ability via overexpression of the *S. cerevisiae* SUT1 gene\(^{55}\) failed in our systems. Therefore, the possibility of introducing truncations in the sterol biosynthetic pathway in order to divert carbon into diterpene biosynthesis had been avoided. The
upc2-1 allele represents an opportunity to incorporate sterol deletions since sterol importation is observed upon upc2-1 incorporation while, at the same time, increase the overall rate of sterol biosynthesis.

To conserve selective markers, the strains carrying the upc2-1 allele, CJ2-A and SC2-1C, were obtained (a generous gift from Prof. L. Parks), and genetically crossed to EHY1. The resulting strains were named EHY18 (MAT\(a\) pGal1-BTS1::his pGal1-trHMG1::LEU2 upc2-1 ura3-52 leu2-3,112 trp1-Δ63 his3-Δ200 ade2 Gal+) and EHY19 (MAT\(α\) pGal1-BTS1::his pGal1-trHMG1::LEU2 upc2-1 ura3-52 leu2-3,112 trp1-Δ63 his3-Δ200 ade2 Gal+). Both strains were transformed with pEH9.0 and grown in inducing media to observe the amount of biosynthesized diterpene. In conjunction, both JBY575[pEH9.0] and EHY1[pEH12.2][pEH9.0] were analyzed to establish, if any, the degree of increased diterpene production. The peak corresponding to abietadiene from EHY18[pEH9.0] indicated an increase of nearly three-fold in diterpene production over EHY1[pEH12.2][pEH9.0] (a 300-fold increase in diterpene production relative to wild type). The uninduced cultures of each strain exhibited undetectable levels of abietadiene by GC analysis.

Because EHY18[pEH9.0] demonstrated a slightly higher diterpene yield (~3 mg/L) than EHY19[pEH9.0] (~1.5 mg/L), the former was further investigated. To determine the effect of increasing the carbon content in the growth media, induced cultures of EHY18[pEH9.0] contained various initial galactose concentrations were tested(Figure 7). The graph illustrates a comparison of abietadiene obtained from induced cultures
containing 2 %, 4 %, and 8 % galactose concentrations. The same strain but lacking cyclase activity, EHY18, was grown under similar inducing conditions to determine product background; expectedly, only EHY18[pEH9.0] produced abietadiene.

The error bars represent the variation observed in three replicate experiments.

Figure 7. 7,13-Abietadiene production upon induction of EHY18[pEH9.0] in various galactose concentrations
The maximum amount of abietadiene obtained in 4 % galactose increased nearly two-fold over the abietadiene detected in 2 % galactose. However, increasing galactose from 4 % to 8 % did not induce a marked increase in the amount of abietadiene detected. The experiment indicated that a two-fold increase in diterpene production results from cultivating with higher amounts of galactose (~ 6 mg/L in 4 % galactose).

Because in vivo diterpene production reached a level suitable for product characterization, a 1 L induced culture of EHY18[pEH9.0] was grown. The resin eluant was purified for the major product (5 mg, 85 % pure) and confirmed by $^1$H-NMR to be biosynthetic 7,13-abietadiene (Figure 8). The abietadiene fraction contained at least three biosynthetic products possessing m/z = 272 by GC/MS analysis. The major compound (97 % relative ratio) was confirmed to be 7,13-abietadiene. An isomer (3 % relative ratio)

![Diagram of abietadiene synthesis](image)

**Figure 8. Product profile of A. grandis abietadiene synthase**

of biosynthetic origin produced a fragmentation pattern that corresponded to neoabietadiene. In addition, NMR data demonstrated an upfield methyl singlet (δ
0.629 ppm) indicated the presence of another isomer possessing, most likely, a double bond at the C-7 position. No evidence of levopimaradiene was found despite significant production of this isomer detected upon expression of a truncated abietadiene synthase and \textit{in vitro} incubation with substrate.\textsuperscript{179}

Optical densities of induced cultures expressing abietadiene synthase, EHY18[pEH9.0], were compared to optical densities of induced cultures lacking the cyclase activity, EHY18. The induced EHY18 and EHY18[pEH9.0]

![Graph showing optical density (OD) vs. % Galactose](image)

\textbf{Figure 9.} Optical density of induced cultures containing various galactose concentrations: EHY18 and EHY18[pEH9.0]
cultures grown in various galactose compositions were measured \((\lambda = 600\,\text{nm})\) for cell
density prior to sample processing (Figure 9). The optical density measurements of
EHY18 and EHY18[pEH9.0] were significantly increased in 4\% inducing media relative
to 2\% inducing media. However, neither strain gained a growth advantage from increasing
to 8\% galactose. Comparing measurements of the two strains in all galactose
concentrations suggests that an adverse effect on growth rate occurred in the absence of
abietadiene activity. The saturation levels differed in contrast to recombinant strains
without \textit{upc2-1}.

Hypothesizing that higher levels of accumulating GGPP contributes to the observed
decrease in growth densities, EHY18 was analyzed relative to EHY18[pEH9.0] for
GGOH content (data not shown). EHY18 excreted undetectable amounts of GGOH in
both 4\% and 8\% galactose but 2\% galactose yielded GGOH amounts equivalent to
those obtained from EHY18[pEH9.0] (2-7\,mg/L extrapolated from GGOH standard at a
known concentration). In contrast, the induced cultures of EHY18[pEH9.0] provided
consistent detection of GGOH, but no correlation existed between these GGOH levels
and the concentration of galactose in the growth media. Throughout the course of
evaluating the EHY18 strains, similar irreproducibility in GGOH levels continued to
plague the results. For example, in one experiment the amounts of detectable GGOH in
EHY18[pEH9.0] reached nearly 40\,mg/L; incidentally, the amount of detectable
abietadiene in that same experiment calculated to approximately 10\,mg/L. The observed
lack of precision suggests that the amount of biosynthesized GGPP \textit{in vivo} cannot be
accurately measured via excreted GGOH levels. Factors possibly contributing to the compromised precision include but are not limited to the variance in hydrolysis of GGPP, exportation of GGOH out of the cell, or the partitioning factor associated with resin adsorption.

The analytical methodology designed to evaluate diterpene yields relied on a number of factors: (1) efficient hydrolysis and translocation of abietadiene and GGPP/GGOH from the cell, (2) quantitative adsorption efficiency of the porous resin, and (3) strain stability under inducing conditions. These assumptions introduce several degrees of freedom into the quantitative values obtained from the methods application. Based on the imprecision in GGOH detection, at least one of the named assumptions is invalid.

Development of the analytical methodology

The analytical method used throughout the construction of the recombinant yeast strains improved compound recovery. Previously, biosynthetic diterpene was extracted directly from the cell lysate and culture media. Because prenyl alcohols of biosynthetic origin have previously been isolated from growth media, we theorized that the accumulating diterpene may also be excreted out of the cell.\textsuperscript{113} Hydrophobic resin is sometimes used to isolate secondary metabolites from cultures in an effort to sequester the compounds, thereby thwarting degradation.\textsuperscript{180,181} A similar method was incorporated to improve compound recovery of excreted abietadiene. A porous polyaromatic resin (Diaion HP-20) was purchased from Supelco.
The resin was (5 % (w/v) of the induced culture) added to a Corex tube that displayed similar dimensions to the sterile polypropylene tubes previously used for culture cultivation. The preparation of the resin included wetting with alcohol followed by sterilization. The wetting procedure was tested with methanol, the recommended solvent, and ethanol. Ethanol was investigated because residual amounts of this alcohol would be better tolerated by the yeast than methanol. However, no effect on diterpene production was observed between the different wetting solvents.

Additionally, optimization of the dilution factor (inoculum volume) and induction time in the presence of the resin was performed. The concentrated inoculum (1:20 dilution factor) yielded no obvious advantages to a more dilute induction volume (1:1000); therefore, the latter was chosen to allow for additional generations under inducing conditions. The induction time was tested at 48, 72, 96, and 105 hours. The yields of detectable diterpene benefited from longer induction times. During these experiments, the culture media and the cell lysate of cultures grown with and without resin were also extracted to ensure that the resin incorporation provided an increase in recovery as compared to the techniques used previously.

The effect of the resin on the optical densities of induced cultures was determined. EHY1[pEH12.2][pEH9.0] was induced for 48 h in the presence and absence of resin. The optical density reached by the culture devoid of resin was twice the optical density reached by the culture containing resin. Although the induced culture lacking resin produced a higher cell mass, this sample (media and cells) contained only 0.03 mg/L abietadiene. In contrast, the same strain which grew to a lower cell density because of the
resin was processed by the empirically established protocol (see Materials and Methods) indicated a 0.7 mg/L level of diterpene production in vivo. The filtrate removed from the resin was also extracted to determine the efficiency with which the excreted abietadiene bound to the resin. An additional 15 % (0.1 mg/L) of the total abietadiene detected from the resin eluant was detected in the extracted culture media. To compensate for binding inefficiency due to short exposure time, all samples described throughout this chapter were grown for four days unless otherwise specified; the longer incubation times did not significantly increase optical density measurements. A control of growth media containing resin (no cells) was included to assign the analytical background contributed by the resin.

Once established, the utility of the resin for purposes of improving recovery of abietadiene was confirmed and, therefore, replaced the previous direct extractions of the culture media and cell lysates. The incorporation of the resin to sequester excreted hydrophobic compounds of biosynthetic origin allowed nearly a seven-fold increase in recovered abietadiene; also, through the use of the resin, we learned that excretion of GGOH occurs upon in situ accumulation of GGPP. The excreted prenyl alcohol adsorbs onto the polyaromatic resin, thereby permitting direct recovery and analysis. Unfortunately, as described above, the correlation between detectable GGOH and biosynthetic production of GGPP in vivo can only be indirectly inferred from this analytical process. The reasons contributing to this variability remain unknown.
Modifications in squalene synthase

The construction of EHY18[pEH9.0] emphasized significant improvement towards producing diterpenes (now in the range of 5 - 10 mg/L). Efforts to further increase the yields continued with the use of a third approach: increasing GGPP biosynthesis by minimizing the metabolic flow to competing biosynthetic processes, namely sterol biosynthesis. The formation of squalene results from the head-to-head condensation of two molecules of farnesyl pyrophosphate (FPP); the two-step transformation constitutes a branch point in the sterol biosynthetic pathway. Commonly, branch point transformations are highly regulated steps in a metabolic pathway, and consequently, squalene synthase (ERG9) regulatory mechanisms have been investigated (see Background). Transcription of ERG9 reportedly decreases upon growth in semi-anaerobic environments. Therefore, the subsequent experiment involved cultivating EHY18[pEH9.0] under semi-anaerobic conditions to investigate the effects of decreased sterol biosynthesis on diterpene production.

The apparatus required 30 mL rather than the previously utilized 5 mL inducing media to minimize head space in the vial, but the polyaromatic resin remained at 5 % (w/v) of the induction media (2 % galactose). Once the inoculum was added (1:1000 dilution factor), the vial was sealed with a screw cap and Teflon-lined septum. The septum permitted removal of the generated CO₂, which was performed using aseptic technique. No effort was made to remove the air initially captured in the head space of the vial, which indicates that the cells initially grew in an aerobic environment. However,
once the residual oxygen was consumed, the cells experienced anaerobic mitotic growth in which ERG9 transcription would be suppressed.

The culture was processed similarly to the 5 mL cultures and analyzed by GC. A calibration curve was used to estimate biosynthetic yield and indicated that 1.4 mg/L abietadiene was obtained under these modified growing conditions. Although the semi-anaerobic growth conditions did increase biosynthetic production of abietadiene relative to wild type yeast, the yields did not surpass those obtained under aerobic conditions (~3 mg/L). Experimental parameters were not optimized; instead, a genetic alternative to decreasing ERG9 activity was pursued.

Temperature-sensitive mutations allow examination of impaired enzymatic activity by partially blocking metabolism. Temperature sensitive mutations weaken an enzyme's secondary structure. The resultant protein becomes especially sensitive to thermal denaturation, thereby rendering its activity temperature sensitive. To investigate how a temperature-sensitive ERG9 mutation affects diterpene production, erg9-1<sup>83</sup> was incorporated into EHY1 by genetic cross. Those strains analyzed contained the erg9-1 temperature sensitive mutation as determined by observing growth at various temperatures relative to EHY1 (control strain).

EHY32 (MATα, pGAL1-trHMG1::LEU2 upc2-1 erg9-1 ura3-52 leu2-3,112 trp1-Δ63 his3-Δ200 ade2 Gal<sup>+</sup>) and EHY36 (MATα, pGAL1-BTS1::LEU2 erg9-1 ura3-52 leu2-3,112 his3-Δ200 ade2 Gal<sup>+</sup>) were each transformed with pEH9.0, and EHY32 was cotransformed with pEH12.2 and pEH9.0 (see Appendix II). Preliminary growth temperature determination was performed on solid media and indicated that growth
temperatures exceeding 36 °C approached lethal levels. Induction of all strains at 30 °C established the theoretical minimum in diterpene production (Table 2). Because growth

Table 2. 7,13-Abietadiene Production in S. cerevisiae Carrying the erg9-1 Allele

<table>
<thead>
<tr>
<th>Thermal Induction Conditions</th>
<th>Days Grown</th>
<th>Amount 7,13-abietadiene (pA)</th>
<th>Amount GGOH (pA)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>EHY32[pEH9.0]</strong> (erg9-1, upc2-1, Gal1p-BTSI)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30 °C</td>
<td>4</td>
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<td>nd</td>
</tr>
<tr>
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<td>8</td>
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</tr>
<tr>
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<tr>
<td>* 34 °C</td>
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<td>nd</td>
</tr>
<tr>
<td>* 34 °C</td>
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<td>39.9</td>
<td>3.2</td>
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<td><strong>EHY32[pEH12.2][pEH9.0]</strong> (erg9-1, upc2-1, Gal1p-BTSI,[trHMG1])</td>
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<tr>
<td>30 °C</td>
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<td>5.0</td>
</tr>
<tr>
<td>* 32 °C</td>
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<td>1.7</td>
<td>4.4</td>
</tr>
<tr>
<td>* 34 °C</td>
<td>7</td>
<td>3.0</td>
<td>24.8</td>
</tr>
<tr>
<td>† 34 °C</td>
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<td>1.2</td>
<td>6.5</td>
</tr>
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<td>30 °C</td>
<td>3</td>
<td>5.5</td>
<td>nd</td>
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<td>34 °C</td>
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<tr>
<td>34 °C</td>
<td>4</td>
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</tr>
<tr>
<td>* 34 °C</td>
<td>2</td>
<td>5.0</td>
<td>nd</td>
</tr>
</tbody>
</table>

nd = not detected. pA indicates the peak area integrated by GC in picoamps.

* denotes an initial 24 h incubation at 30 °C followed by incubation at the designated temperature for the number of days indicated.

† denotes 24 h incubation at 30 °C followed by a 24 h incubation at 32 °C prior to the incubation designated.

of all erg9-1 strains lagged even at 30 °C, both EHY32 and EHY36 benefited from longer incubation times. At higher growth temperatures, an initial 24 h incubation at 30 °C
(denoted with an *) was determined to be advantageous to diterpene production in EHY32[pEH9.0]. The inconsistent incubation times resulted from various growth rates observed per strain. Initially, the cultures were processed based on a predetermined incubation time. However, induced EHY32[pEH9.0] cultures grown at 30 °C for 2 days, 34 °C for 4 days, and * 34 °C for 2 days yielded optical densities of 1, 2.4, and 1.7, respectively. These values indicate that the incubation times require lengthening. Therefore, the remaining cultures were processed after a visual determination of saturation rather than a predetermined incubation time.

Overexpression of truncated HMG1 (pEH12.2) in EHY32 yielded an increase in detectable GGOH suggesting that an increase in the native metabolic rate of sterol biosynthesis is critical for GGPP/GGOH accumulation. However, the same strain produced lower levels of detectable abietadiene than the analogous strain lacking pEH12.2. In EHY32[pEH12.2][pEH9.0], the unchanged levels of detectable abietadiene accompanied by a concomitant increase in detectable GGOH hints at an adverse effect on GGPP cyclization with increasing growth temperatures. Furthermore, EHY36 lacked both genetic pieces that facilitated a metabolic flux increase and, consequently, produced relatively little abietadiene and no detectable GGOH.

Of the three genotypes analyzed, EHY32[pEH9.0] grown at 34 °C produced the highest amount of detectable abietadiene (approximately 1.5 mg/L). However, this strain did not produce the maximum amount of detectable GGOH; the additional expression of pEH12.2 allowed approximately 0.6 mg/L GGOH upon cultivation at 34 °C. Neither
yield represents a significant increase in diterpene metabolism relative to
EHY18[pEH9.0]. The incorporation of a temperature-sensitive mutation in ERG9
proved unsuccessful with respect to increasing diterpene production in vivo.

**Ergosterol production in metabolically engineered yeast**

A final experiment was performed to determine the amount of ergosterol biosynthesized
in the recombinant yeast strains known to produce diterpenes (Table 3). The ergosterol
levels indicate the portion of carbon that has been redirected away from ergosterol
biosynthesis and to what degree that level affected production of downstream sterol
intermediates. Therefore, induced cultures of JBY575[pEH9.0],
EHY1[pEH12.2][pEH9.0], EHY18[pEH9.0], and EHY32[pEH9.0] were evaluated for
total ergosterol content. Saponification of 50 mL induced cultures not containing resin
afforded approximately 1 mg total sterol. Silylation of the reaction products followed by
GC analysis compared to silylated ergosterol prepared at known concentrations afforded
the results shown in Table 3. Wild type yeast expressing pEH9.0 established the value
for native sterol biosynthesis at 1.6 mg/mL. The sterol metabolic flux increased in
response to overexpressing truncated HMG1 and incorporating upc2-1. Carbon was
successfully diverted to produce GGPP and abietadiene. Neither overexpressing BTS1,
truncated HMG1, nor the incorporation of erg9-1 were as effective in increasing sterol
metabolism as upc2-1 incorporation.
The result confirms the previously reported increase in ergosterol biosynthesis.

However, the reported accumulating ergosterol was found as the acyl ester. In this

Table 3. Ergosterol content in recombinant *S. cerevisiae* producing 7,13-Abietadiene

<table>
<thead>
<tr>
<th>STRAIN</th>
<th>ERGOSTEROL (mg/mL)</th>
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<tbody>
<tr>
<td>JBY575[pEH9.0]</td>
<td>1.6</td>
</tr>
<tr>
<td>EHY1[pEH12.2][pEH9.0]</td>
<td>1.8</td>
</tr>
<tr>
<td>EHY18[pEH9.0]</td>
<td>7.4</td>
</tr>
<tr>
<td>EHY32[pEH9.0]</td>
<td>1.3</td>
</tr>
</tbody>
</table>

experiment, saponification allowed hydrolysis of accumulating ergosteryl ester and subsequent detection of both free and esterified ergosterol. The 4.5-fold increase in ergosterol content exhibited by EHY18[pEH9.0] directly reflects the increase manifested by upc2-1. Moreover, this excess implies that more carbon from sterol biosynthesis can be redirected to diterpene production *in vivo* than presently being exploited by EHY18[pEH9.0]. The potential amount of accessible carbon for diterpene production proves a tantalizing target for future modifications and leaves this project in a position assuring its utility in diterpene production.
CHAPTER 4: RESULTS AND DISCUSSION

Sesquiterpenes: in vivo Production in Recombinant S. cerevisiae

This chapter explores metabolic engineering of yeast as a production system to biosynthesize sesquiterpenes. The steps investigated lay a foundation towards the design of a feasible sesquiterpene production strain. Recombinant strains efficient in sesquiterpene production can serve as competitive alternatives to produce and consequently access sesquiterpenes. Potential physiological implications as well as proposed future work are discussed.

Sesquiterpene production in wild type yeast

To establish the amount of sesquiterpene precursor, FPP, that could be funneled out of sterol metabolism for purposes of sesquiterpene production, the wild type yeast JBY575 (MATa ura3-52 trp1–Δ63 leu2-3,112 his3-Δ200 ade2 Gal+) was transformed with pLH4.12. This plasmid contains a sesquiterpene cyclase cloned from Artemisia annua that produces epicedrol\textsuperscript{32} (Figure 4) in a multiple copy yeast expression vector pRS426Gal. The cyclase serves as the second model compound used to illustrate the efficiency of each recombinant strain developed in this project. The first model utilized Fusarium sporotrichioides trichodiene synthase,\textsuperscript{148} but undetectable levels of trichodiene were consistently observed. The possibility existed that trichodiene may possess inherent cytotoxicity to yeast. After the epicedrol synthase became available, incorporation of this cyclase was performed to confirm the authenticity of the negative
results. The resulting strain, JBY575[pLH4.12], produced approximately 0.1 mg/L epicedrol (based on authentic standard measured at a known concentration) as determined by GC and GC/MS analysis (m/z 220). From this point forward, all genetic and metabolic modifications were analyzed for detectable epicedrol. All samples result from the elution of a polyaromatic resin that resides in the induction media.

Wild type yeast biosynthesize FPP at levels higher than native GGPP biosynthesis. As a result the detectable epicedrol observed in unmodified yeast reached amounts equivalent to EHY1[pEH9.0], which contained a genetic modification to increase protein levels of the GGPP synthase. Because squalene biosynthesis poses the largest consumption of FPP, squalene synthase (ERG9) modifications were investigated. Two external transcriptional control approaches were explored: copper inducible squalene synthase and temperature-sensitive squalene synthase.

Modifications in Squalene Synthase and Farnesyl Pyrophosphate Synthase

Most eukaryotes produce a group of low molecular weight, cysteine-rich polypeptides that scavenge and sequester heavy metal ions. In S. cerevisiae, the copper metallothionein proteins encoded by CUP1 and CRS5 exhibit negative regulation by oxygen. Heterologous expression of genes under control of the CUP1 promoter yielded competitive transcriptional levels relative to the strong, constitutive GAPDH promoter and allowed successful characterization of a mammalian reductase. Based on the reported information, a construct was prepared that incorporated the CUP1
promoter into the chromosomal ERG9 via homologous recombination. The resulting strain, EHY9, contained a lesion in porphyrin biosynthesis that once corrected yielded EHY10. Transformation of this strain with pLH4.12 yielded EHY10[pLH4.12]; cultures were cultivated in the presence of exogenous copper (250 μM)\(^{185}\) to maintain ERG9 transcription levels and consequently an intact sterol biosynthetic pathway.

Prior to analysis, truncated HMG1 under control of the GAL1 promoter was incorporated to EHY10. The resulting strain, EHY13, contained two recombinant chromosomal genes: extrachromosomal truncated HMG1 and ERG9 under transcriptional control of CUP1 rather than native control. EHY13 was transformed with pLH4.12. Preliminary copper sensitivity investigations yielded epicedrol production of approximately 0.05 mg/L in EHY13[pLH4.12] grown with no added copper (yeast extract contributes 0.3 μM copper). The amount constitutes a decrease in sesquiterpene production in vivo relative to wild type. In this same sample, excreted farnesol was detected. The accumulating biosynthetic FPP estimated approximately 0.4 mg/L (authentic standard at a known concentration). Addition of copper (250 μM) to both EHY10[pLH4.12] and EHY13[pLH4.12] provided undetectable levels of epicedrol and a ten-fold decrease in farnesol excretion in EHY13[pLH4.12]. The amount of detectable farnesol proved independent of exogenous copper concentrations as determined by induction of EHY13[pLH4.12] in copper levels covering a 10\(^2\) concentration range. Perhaps the ineffectiveness of the CUP1 promoter system results from the presence of only one copy of the heterologous construct.\(^{186}\) Nonetheless, the data suggest that the host strains efficient copper detoxification machinery displays high sensitivity rendering
the pCup1-ERG9 inefficient at decreased ERG9 transcription.

Prior to pursuing the second approach to down-regulating ERG9, upc2-1 incorporation was tested. Heterologous expression of ERG20 under control of the GAL1 inducible promoter in the host strain CJ2-A,59 which contained the upc2-1 allele, afforded similar epicedrol levels to EHY13[pLH4.12] and undetectable levels of farnesol. Further modification of this strain, EHY24 (MATa pGal1-ERG20::LEU2 upc2-1 ura3-52 leu2-3,112 trp1-Δ63 his3-Δ200 ade2 Gal+), improved epicedrol production. Because overexpression of truncated HMG1 proved useful to increase diterpene production, the same modification to EHY24 was investigated. The resulting strain, EHY24[pEH12.2][pLH4.12], produced epicedrol at approximately 0.3 mg/L. Based on the latter result, the overexpression of ERG20 effects a negligible increase in detectable sesquiterpene. Because FPP biosynthesis represents a major branch point in sterol metabolism, the *S. cerevisiae* ERG20 protein probably induces one or more regulatory mechanisms involved in maintaining endogenous ergosterol levels. Should protein degradation constitute one of the regulatory mechanisms, incorporation of heterologous FPP synthases from other eukaryotes may provide a means of avoiding such metabolic control.

Reasoning that *S. cerevisiae* efficiently processes its own enzymes, manipulation of enzymes indigenous to the host have been described. Maintaining this dictum prevented the possibility of translational problems such as codon bias and recognition of false start sites. However, incorporating genetic information from another eukaryote may thwart inherent regulatory mechanisms that initiate when native proteins reach abnormal levels.
Benefits of incorporating genes from alternative organisms must be empirically
determined; the A. thaliana HMG1 gene\textsuperscript{174} was expressed in EHY24 in parallel with an
induced culture of the same strain containing the S. cerevisiae truncated HMG1. The A. thaliana
HMG1 protein lacks a hydrophobic N-terminal sequence but apparently
contains two different transcriptional start sites.\textsuperscript{187} The expression of A. thaliana
HMG1 under control of the inducible GAL1 promoter yielded detectable epicedrol;
however, the production observed afforded statistically equivalent epicedrol levels
relative to EHY24[pEH12.2][pLH4.12]. This data indicates that the truncation of the
yeast reductase is sufficient for exploiting the contribution that increasing HMG activity
can make towards sesquiterpene production.

Because the copper inducible ERG9 exhibited a negative result towards increasing
sesquiterpene production \textit{in vivo}, a different modification was investigated. Altering
protein stability via temperature-sensitive mutations affords a means to manually control
enzymatic activity. Reasoning that such a mutation in ERG9 would provide an
opportunity to decrease the large portion of biosynthetic FPP used for sterol production,
a temperature-sensitive ERG9 mutant (\textit{erg9-1}) was introduced into EHY24. A genetic
cross between \textit{erg9-1} and EHY24 yielded strains containing a combination of genetic
modifications (Table 4).

The highest thermal conditions in which each strain could sustain growth were
predetermined on solid media (see \textit{Experimental}, p.65). However, growth behavior did
not directly correlate to liquid media; the appropriate thermal conditions decreased by 2 -
4 °C. The reported peak areas do not present a significant improvement in detectable
epicedrol or farnesol. A decrease of ERG9 activity would intuitively effect a growing pool of biosynthetic FPP.

<p>| Table 4. Epicedrol Production in <em>S. cerevisiae</em> Carrying the <em>erg9-1</em> Allele |
|----------------------------------|-----------------------------|-----------------------------|-----------------------------|</p>
<table>
<thead>
<tr>
<th>Strain</th>
<th>Genetic Modifications</th>
<th>Maximum Detectable Epicedrol (pA)</th>
<th>Maximum Detectable Farnesol (pA)</th>
<th>Thermal Conditions Obtained</th>
</tr>
</thead>
<tbody>
<tr>
<td>EHY31</td>
<td>MATα <em>erg9-1, upc2-1</em>, <em>pGAL1-trHMG1</em></td>
<td>4.8</td>
<td>17</td>
<td>32 °C (7 d)</td>
</tr>
<tr>
<td>EHY33</td>
<td>MATα <em>erg9-1, upc2-1</em>, <em>pGAL1-ERG20</em></td>
<td>0.7</td>
<td>1.5</td>
<td>32 °C (10 d)</td>
</tr>
<tr>
<td>EHY34</td>
<td>MATα <em>erg9-1, upc2-1</em>, <em>pGAL1-ERG20</em></td>
<td>nd</td>
<td>nd</td>
<td>n/a</td>
</tr>
<tr>
<td>EHY35</td>
<td>MATα <em>erg9-1, upc2-1</em></td>
<td>0.5</td>
<td>3.2</td>
<td>32 °C (6 d)</td>
</tr>
<tr>
<td>EHY37</td>
<td>MATα <em>erg9-1</em></td>
<td>11.7</td>
<td>5</td>
<td>*32 °C (10 d)</td>
</tr>
</tbody>
</table>

*nd* = not detected under any combination of thermal conditions. *n/a* = not applicable. * denotes induced culture grown in 4% galactose. The incubation times are given in parentheses.

The data observed in two different approaches to exploit accumulating FPP afforded similar results in that generally a decrease in the amount of FPP redirected towards sesquiterpene production was observed. In fact, EHY37 defined the only strain manipulated at ERG9 to increase epicedrol production beyond those levels obtained from wild type. Throughout the course of determining thermal conditions, relative comparisons of the different genotypes and the levels of epicedrol detected illuminated the following points of discussion.

First, the increase in HMG1 activity effected an increase in excreted farnesol.
EHY31[pLH4.12] produced the highest detectable levels of farnesol among all of the recombinant strains developed for increased sesquiterpene production. This strain grown at 30°C for 9 d produced undetectable levels of excreted farnesol suggesting that the presence of \textit{erg9-1} contributed significantly to the observed farnesol levels upon induction at 32°C. Second, the ERG9 mutation alone cannot sufficiently account for the amount of farnesol found in EHY31 because maximum levels in EHY37 reached only 30% of the farnesol detected in EHY31. Furthermore, EHY37 cotransformed with pEH12.2 and pLH4.12 produced epicedrol levels equivalent to wild type (data not shown). This result indicates that the overexpression of truncated HMG1 adversely affected the epicedrol production levels in EHY37. Understanding this result requires further investigation of \textit{erg9-1}.

Third, overexpression of ERG20 appeared to demonstrate a negative effect on both sesquiterpene and farnesol production. Review of the literature suggests that ERG20 activity and consequent FPP levels may be determined by an unidentified independent regulatory network (see Background, p. 21). In addition, the increase in dolichol biosynthesis observed upon ERG20 overexpression in yeast mutant in squalene synthase (\textit{erg9}) suggests that a sink for accumulating FPP already existed. The approach used in this work assumed that by installing an artificial tributary (cyclization to epicedrol), redirection of FPP towards sesquiterpene production could be enhanced. In two perspectives the approach succeeded: increased sesquiterpene production was observed but not near commercially feasible levels, and many intriguing questions have been raised by the observations described.
The fourth observation indicates that a-factor yeast present a problem when used as a host for sesquiterpene production. EHY34 was unable to grow under inducing conditions. This strain exhibits the same genotype as EHY33 with the exception of its mating type. One of the a-factor mating pheromones requires covalent bond formation with one molecule of FPP prior to secretion. EHY34 represents the single \textit{erg9-1} strain obtained of eight tested (including those developed for diterpene production) that possess the a-mating type. The inability to cultivate EHY34 in the presence of galactose may be linked to the insufficient FPP available for protein prenylation and/or specifically mating factor alkylation. Two plausible solutions exist for avoiding the possible a-factor complication: (1) maintain a \textit{MAT\alpha} host, or (2) use diploid strains. The successful carotenoid production in metabolically engineered \textit{C. utilis}, the diploid food yeast, exemplifies an example of the latter.\textsuperscript{4}
Metabolically engineered *S. cerevisiae* represents a feasible production system for diterpenes and possibly sesquiterpenes. Consequently, such recombinant strains can demonstrate improvement over current microbial host for cloning and functional characterization of plant cyclases. Plant diterpene production occurs in the plastid organelle and primarily utilizes the ancient deoxyxyulose pathway as a resource for IPP. Translocation of the diterpene cyclase from the cytosol requires a hydrophobic plastidial transit peptide. To complicate matters, these signal sequences normally contain several hydrophobic residues causing the formation of inclusion bodies upon bacterial expression. A yeast strain that can efficiently express unmodified cyclases (as preproteins) circumvents removal of the transit peptides. For example, abietadiene synthase, the diterpene cyclase used in this thesis work, is blocked at the N-terminus, thereby preventing protein sequencing. Prior to cloning and characterization, a soluble form of the protein had to be constructed. However, expression of the full-length gene in the recombinant yeast strains described in this section did not require such modifications. Although the full-length abietadiene synthase became rate-limiting in the biosynthesis of diterpenes (and could benefit the biosynthetic yields by being truncated), enough enzymatic product was produced by the full-length protein to allow for functional characterization of the enzyme. Therefore, the described engineered yeast strains can facilitate functional characterization of diterpene cyclases especially those bearing hydrophobic terminal sequences.
Part II:

Characterization of Sterol Biosynthetic Enzymes
CHAPTER 6: INTRODUCTION

Molecular phylogeny explores the origin of an organism by evaluating its genetic information\(^{189}\) as opposed to its physical and morphological characteristics. Within a decade of the introduction of this method, molecular phylogeny changed a longstanding scientific dogma: the living world now existed as three, rather than two, primary domains.\(^{190-192}\) The discovery of the Archaea Kingdom exemplified the significant contribution that molecular phylogeny could offer scientists in unraveling relationships in Nature.

Woese's technique of comparing sequences encoding single-subunit ribosomal RNAs (SSU rRNA) not only established a third domain but also validated the endosymbiont hypothesis\(^{193}\) and demonstrated that eukaryotes originated from archaea.\(^{194}\) Curiously, the origin of many eukaryotic nuclear genes found closer resemblance to bacteria rather than archaea; presently, lateral gene transfer, known to be common among bacteria, represents the most likely reason for these incongruent results.\(^{195}\) From identifying a new kingdom to exploring horizontal gene transfer among all three phylogenetic domains, molecular phylogeny has continued to illuminate the origins of Life.

Additionally, molecular phylogeny has contributed significantly to advances in biochemical research. Sequence homology and sequence identity methods established by Woese have become irreplaceable tools in the study of enzymatically-assisted chemical processes. These techniques guide prediction and investigation of potential active-site residues through genomic comparisons. Although active-site residues are generally highly
conserved, sequence comparisons based solely on residues that chemically interact with the substrate are unrealized. Therefore, the methods presently rely on crystal structure and mutageneic data to facilitate catalytic residue assignments.

In particular, the following section describes the identification of a catalytic residue in oxidosqualene cyclases. However, the residue demonstrates differential conservation among eukaryotes, thereby thwarting assignment based exclusively on sequence alignments. By adding this functional comparison dimension, genomic comparison methodology demonstrates an increased utility towards assigning catalytic residues to proteins. Consequently, the described experiments establish a correlation between genetic and chemical differences among eukaryotes.
Part II: Background

Almost all eukaryotes probably require sterols to sustain life, but not all eukaryotes are capable of sterol biosynthesis. Insects such as Drosophila pachea, a fruit fly found in arid biomes, represent examples of eukaryotes that cannot biosynthesize sterols. However often through symbiotic relationships with other eukaryotes, insects obtain sterols which are biochemically necessary for their life. The focus of this thesis will be limited to comparing higher eukaryotes that can biosynthesize sterols de novo, specifically, fungi and plants.

Efforts to delineate the biochemical processes controlling specific transformations and to determine variations of those processes among eukaryotes require an understanding of sterol metabolism. In eukaryotes, sterol biosynthesis proceeds through common intermediates up to the formation of (S)-2,3-oxidosqualene (OS). The subsequent transformation, cyclization of OS, represents a key difference between plants and fungi and animals. This catalytic divergence constitutes the site of this investigation into genetic and chemical differences among eukaryotes.

Epoxidation of Squalene Leading to the Formation of Sterols

Oxidosqualene (OS) results from epoxidation of squalene (see Part I, Background) at the C2-C3 position. This reaction is catalyzed by the mono-oxygenase ERG1 and represents the drug target for topical antifungal ointments. Subsequently, cyclization of OS effects the production of lanosterol which is catalyzed by
lanosterol synthase (ERG7).\textsuperscript{175,201} Any enzyme that catalyzes the cyclization of OS is denoted as an oxidosqualene cyclase (OSC).\textsuperscript{202} For example, plants cyclize OS to the pentacyclic isomer of lanosterol known as cycloartenol.\textsuperscript{203} The \textit{Arabidopsis thaliana} cycloartenol synthase, CAS1, is used as the model plant OSC in this thesis work\textsuperscript{204} although other plant cycloartenol synthases have also been cloned.\textsuperscript{205-207}

The biochemical mechanism of cyclization to produce lanosterol and cycloartenol proceeds through a protosteryl cation (Figure 10). "Protosterol" refers to a tetracyclic sterol in which no atomic migrations have taken place\textsuperscript{208}, as shown in Figure 10 as the first cyclized intermediate. OS must adopt a chair-boat-chair configuration prior to cyclization to yield the protosteryl cation intermediate required to form yield either lanosterol in fungi and animals or cycloartenol in plants.\textsuperscript{200} Presumably, the OSC enzyme positions the substrate in the exact spatial configuration required to effect facile ring closures. Wagner-Meerwein rearrangements\textsuperscript{199,209} occur to reposition the carbonium ion onto C-9.\textsuperscript{202} Although the biochemical processes directing these migrations remain undefined, the product's stereochemistry results from these atomic shifts.

Once the lanosteryl cation forms, deprotonation neutralizes the positive charge at C-9. Deprotonation occurs at C-8 to form a double bond at the C-8 position (lanosterol) or at C-19 to produce the 9β,19-cyclopropyl ring (cycloartenol). The difference in deprotonation sites constitutes the sole catalytic difference between lanosterol
Figure 10. Cyclization of (S)-2,3-oxidosqualene to 4,4-dimethyl sterols in eukaryotes

formation in animals and fungi and the cycloartenol formation in plants. Furthermore, varying other steps in this complicated sequence (such as substrate folding) generates isomeric cyclization products; to date, nearly one hundred structurally different triterpene alcohols have been isolated in Nature.

During the development of this thesis work, the crystal structure of Alicyclobacillus acidocaldarius squalene-hopene cyclase, SHC, was reported by Wendt et al. Briefly, bacterial SHCs cyclize squalene to pentacyclic hopanes. Similar to oxidosqualene cyclases, the substrate undergoes folding configurations, ring closures, and rearrangements in the active-site cavity of the respective SHC enzyme. Although S. cerevisiae ERG7 shares only 16% sequence identity with the A. acidocaldarius squalene-hopene cyclase, residues implicated in catalysis are among the few conserved residues. Efforts to determine the residue responsible for deprotonation in both OCSs and SHCs using homology-based alignments continue to be described. Such experiments lend
credence to sequence homology and, as a whole, to molecular phylogeny as a tool to elucidate catalytically relevant residues.

In eukaryotes, the formation of 4,4-dimethyl sterols such as lanosterol or cycloartenol commits the pathway to the biosynthesis of end product. In yeast, the intermediates formed distal to lanosterol result from a series of oxidations, reductions, and demethylations to yield ergosterol. Similar processes occur in plants in order to metabolize cycloartenol to sitosterol and stigmasterol (Figure 1), the primary sterol structures found in plant membranes.

Cycloartenol metabolism requires the opening of the highly strained cyclopropyl ring. Previously, the chemical process used by plants to transform pentacyclic sterol to tetracyclic sterol was inferred from in vitro incubations of higher plant microsomes because the cyclopropyl isomerase was difficult to purify and the gene had not been cloned. Learning the exact spatial orientation assumed by cyclopropyl sterols may contribute to understanding their physiological significance. Recently, a flat molecular configuration was assigned to cyclopropyl sterols. This configuration contradicts previous predictions of an arc-shaped molecule.

Additionally, investigation of the chemical mechanism by which a cyclopropyl ring efficiently isomerizes to a double bond in vivo may lead to understanding the biochemical relevance of cyclopropyl sterols. Because the analogous non-enzymatic reaction requires extremely harsh conditions (10% sulfuric acid in refluxing toluene) and produces a myriad of products, the biochemical processes affecting enzymatic conversion (performed at physiological pH and produces a single isomer) are unique chemical transformations.
Cyclopropyl isomerase inhibition studies *in vitro* suggest that stabilization by an active-site residue of the C-9 carbonium ion resulting from acid-catalyzed ring opening is critical for enzymatic activity.\textsuperscript{225,226} However, inhibitors such as 2-aza-2,3-dihydrosqualene, which efficiently inhibit OSCs, exhibit no effect on cyclopropyl isomerase activity.\textsuperscript{219} Yeast does not cyclize OS to cyclopropyl sterols as plants do, and consequently lack cyclopropyl isomerase activity.\textsuperscript{227} To investigate this unique step of sterol biosynthesis in plants, this thesis describes the characterization of the first cloned cyclopropyl isomerase gene.
CHAPTER 7: EXPERIMENTAL

Methods

Small scale In vitro Assay for Triterpene Alcohol Production

A fresh colony of recombinant yeast (containing OSC in pRS426Gal) was inoculated in 5 mL non-inducing medium (2 % dextrose, 2 % synthetic complete medium lacking uracil, and any nutritional supplements required for viability). The culture was allowed to grow at 30 °C to saturation. The cells were then collected by centrifugation (1300 × g, 5 min), rinsed with 500 μL sterile deionized H₂O, and resuspended in 5 mL sterile deionized H₂O. One hundred microliters of the cell suspension was used as inoculum for 5 mL inducing media (2% galactose, 2% synthetic complete medium lacking uracil, and additional nutritional supplements required for viability). The induced culture was allowed to grow at 30 °C to saturation. The culture was transferred in equal volumes to tared microfuge tubes, and the cells were collected by centrifugation (14,000 × g, 5 min). The supernatant was decanted, and the wet cell weight was taken. To resuspend the cells, 100 μL buffer (0.1 M NaPO₄, pH 6.4) was added to yield a 20% slurry. Acid-washed glass beads were added, and the cell suspensions were mixed by vortex to lyse the yeast cells. A short incubation on ice cooled the suspension, then substrate was added from a 20 × stock (200 mg (±)-oxidosqualene and 2 g Triton X-100 dissolved in 2 mL methylene chloride; organic solvent was completely removed in vacuo, and 8 mL deionized H₂O was added followed by mixing to homogeneity). A cell suspension
without substrate served as a control.

Reactions were monitored by TLC (3 developments with diethyl ether followed by a single development in 1:1 hexane/diethyl ether). Enzymatic product was visualized with p-anisaldehyde (0.02 mM $p$-anisaldehyde in 15 mL acetic acid, 50 mL concentrated $\text{H}_2\text{SO}_4$, 1350 mL ethanol) and compared to authentic standards of ergosterol and lanosterol (representing 4,4-dimethyl sterols).

**Analytical Instrumentation and Methods**

All instruments used are the same as those described for Part I. Specific analytical methods for triterpenes are described.

*GC analysis.* The oven program designed for derivatized triterpenes used an isothermal oven program at 280 °C; injector was set to 280 °C, and the detector was held at 290 °C. Cholesteryl acetate (1 mg/mL) served as internal standard, and standards for cholesterol and ergosterol were purchased from Sigma Chemical Company. Prof. J.-L. Giner generously provided authentic standards of cycloecaleenal and obtusifoliol.

Triterpenes were analyzed as acetates or tetramethylsilyl ethers. Acetylation was performed by dissolving the triterpene alcohol in methylene chloride to 0.2 mol/L per reaction. Once dissolved, acetic anhydride (5 mmol), triethylamine (5 mmol), and dimethylaminopyridine (DMAP, 0.1 equiv.) were added; the reaction was stirred at ambient temperature until $\geq 90\%$ complete as determined by TLC (10% ethyl acetate in hexane). The reaction was quenched with the addition of deionized $\text{H}_2\text{O}$. The reaction
was extracted thrice with hexane then washed thrice with saturated CuSO₄. The crude acetylated triterpenes were chromatographed (1% ethyl acetate in hexane) prior to GC analysis. Silylation was performed by combining 1-2 mg terpene alcohol with 100 mL 1:1 pyridine/bis(trimethylsilyl)trifluoroacetamide. After mixing, the reaction was allowed to proceed for 1 hour at 40°C. GC analysis was performed after condensing the silylation reaction to near dryness under a stream of nitrogen then reconstituting the derivatized products in toluene.

**GC/MS.** GC/MS analysis was performed on VG ZAB-HF GC-MS. The oven programs were those described above for the respective terpene class, and the column used was J&W Scientific DB-5ms (60 m × 0.25 mm i.d., 0.1 mm df). Transfer lines were set to the same temperature as the injector. Ionization by electron-impact was achieved at 70 eV and scan time of 4.0 sec.

**¹H-NMR and ¹³C-NMR.** Nuclear magnetic resonance (NMR) experiments were done on a Bruker AMX500 or Avance spectrometer (500.1 MHz for ¹H) equipped with a 5-mm inverse-geometry broadband probe. ¹H NMR spectra were measured at 25°C in CDCl₃ solution (generally 5 to 20 mM) and referenced to internal tetramethylsilane. ¹³C NMR spectra were measured at 25°C in CDCl₃ solution (5 to 150 mM) and referenced to CDCl₃ at 77.0 ppm. NMR samples (0.5 mL in 507-PP or 528-PP tubes from Wilmad Glass Co.; Buena, NJ) were shimmed with spinning at 16-20 rps to give a narrow (W₁/₂ @ 0.5 Hz), symmetrical line for tetramethylsilane. CDCl₃ (Cambridge Isotope Laboratories;
Andover, MA) was 99.8 % D.

**Experimental Procedures**

*Arabidopsis thaliana CAS1 I481V*

*Isolation of Enzymatic Products produced by A. thaliana CAS1 I481V.* A saturated 3 L induced culture of SMY8[CAS1 I481V] was transferred to tared Nalgene bottles, and cells were collected by centrifugation (8000 × g, 15 min, 4 °C). The wet cell weight was calculated (~ 47 g) and 59 mL chilled buffer (0.5 M NaPO₄, pH 6.4) was added to adjust the cells to ~ 40 % (w/v) slurry. The cells were lysed by three passes through a French Press (20,000 psi). An additional 100 mL chilled buffer was added to the lysed cells to adjust to ~20 % (w/v) slurry, and 10 mL substrate (substrate stock solution described in Small scale in vitro assay for triterpene alcohol production, p. 109) was added. The reaction was allowed to incubate at ambient for 16 h (~ 50 % complete by TLC) then quenched with 400 mL ethanol.

The reaction was transferred to a round-bottomed flask and evaporated to nearly half volume by rotary evaporation. To adsorb the detergent used in the reaction, pellicular silica (~ 1 g, 70-230 mesh) was added, and the remaining volume was removed in vacuo. The silica powder was transferred to a chromatograph column; the organic compounds were eluted from the silica with diethyl ether. The ethereal extracts were combined, dried over MgSO₄, and concentrated by rotary evaporation. To isolate the
triterpene products, 93 mg of crude material was subjected to column chromatography (15 % diethyl ether in hexane). Acetylation and flash chromatography of the crude reaction was performed as described in Methods (see p. 110). GC analysis (internal standard = 1 mg/mL cholestane) indicated the presence of three isomers (RRT = 2.34, 2.54, 2.62) in the relative ratio of 52:26:22 resulted from the enzymatic conversion of OS by the CAS1 I481V enzyme. These isomers were identified as cycloartenol, lanosterol, and parkeol, respectively, by the following procedures.

Standards of lanosteryl acetate and cycloartenyl acetate were obtained from 1 L cultures of JBY575 and SMY10, respectively, using the same procedure as outlined for SMY8[CAS1 I481V]. Based on spectroscopic information using these biosynthesized standards, two of the enzymatic products were identified as lanosterol and cycloartenol. The third isomer was isolated by medium pressure argentlc chromatography (gradient 10-20 % toluene in hexane at a rate 1 % / 40 mL) followed by preparative argentlc TLC. Preparative plates (Whatman, SiO₂ 60, 20 cm × 20 cm) were prepared by submerging in saturated methanolic AgNO₃ solution and drying for 15 min at 100 °C; this cycle was repeated 3 times. Three components (Rₜ = 0.62, 0.78, 0.91) were visualized upon staining a 2 cm segment with p-anisaldehyde (0.02 mM p-anisaldehyde in 15 mL acetic acid, 50 mL concentrated H₂SO₄, 1350 mL ethanol; stored at 4 °C). GC analysis of Rₜ = 0.62 product demonstrated that the unidentified isomer was ~96 % pure. ¹H-NMR, ¹³C-NMR, GC/MS, DEPT-135, HSQC, HMBC, NOE and COSYDEC analyses were performed; the product was unambiguously identified as parkeyl acetate.

¹H-NMR (500 MHz; 5 mM solution in CDCl₃, 25 °C): δ 0.644 (d, J = 0.8 Hz, 3H, H-
18), 0.736 (d, J = 0.8 Hz, 3H, H-30), 0.865 (d, J = 0.3 Hz, 3H, H-28), 0.890 (s, 3H, H-29), 0.895 (d, J = 6.6 Hz, 3H, H-21), 0.973 (dd, J = 12.0, 2.3 Hz, 1H, H-5α), 1.038 (dddd, J = 13.3, 10.2, 8.7, 4.9 Hz, 1H, H-22S), 1.068 (d, J = 0.8 Hz, 3H, H-19), 1.289 (m, 1H, H-16β), 1.330 (dddd, J = 13.0, 12.7, 4.2 Hz, 1H, H-15α), 1.335 (m, 1H, H-7α), 1.363 (m, 1H, H-15β), 1.387 (m, 1H, H-20), 1.434 (m, 1H, H-22R), 1.477 (qd, j = 12.4, 3.3 Hz, 1H, H-6β), 1.542 (tdd, J = ~ 13, 3.5, 0.8 Hz, 1H, H-1α), 1.604 (dtd, J = 1.4, 0.8, 0.4 Hz, 3H, H-27), 1.605 (dddd, J = 9.8, 9.3, 8.2 Hz, 1H, H-17α), 1.659 (m, 1H, H-7b), 1.673 (m, 1H, H-6α), 1.678 (qd, J = 12.8, 3.5 Hz, 1H, H-2β), 1.684 (dtd, J = 1.4, 1.2, 0.4 Hz, 1H, H-26), 1.764 (dq, J = 12.6, 3.7 Hz, 1H, H-2α), 1.794 (dt, J = 12.7, 3.5 Hz, 1H, H-1b), 1.860 (m, 1H, H-23R), 1.893 (dddd, J = 13.1, 9.8, 9.1, 6.8 Hz, 1H, H-16α), 1.900 (dddd, J = 17.1, 6.2, 2.3 Hz, 1H, H-12α), 2.051 (s, 3H, CH₃CO), 2.165 (m, 1H, H-8b), 4.483 (dd, J = 11.6, 4.3 Hz, 1H, H-3α), 5.099 (t of septet, J = 7.1, 1.4 Hz, 1H, H-24), 5.228 (dtd, J = 6.3, 2.0, 0.6 Hz, 1H, H-11).

¹³C-NMR (125 MHz, 30 mM solution in CDCl₃, 25°C): δ 14.40 (C18), 16.79 (C-29), 17.63 (C-27), 18.29 (C-21), 18.47 (C-30), 21.23 (C-6), 21.33 (CH₃CO-), 22.29 (C-19), 24.15 (C-2), 24.93 (C-23), 25.71 (C-26), 28.00 (2C, C-7 & C-16), 28.17 (C-28), 33.91 (C-15), 35.79 (C-1), 35.92 (C-20), 36.38 (C-22), 37.13 (C-12), 37.99 (C-4), 39.23 (C-10), 41.74 (C-8), 44.28 (C-13), 47.00 (C-14), 50.91 (C-17), 52.56 (C-5), 80.87 (C-3), 115.18 (C-11), 125.22 (C-24), 130.92 (C-25), 148.11 (C-9), 170.92 (CH₃CO-).

GC/MS: m/z = 468 (1), 408 (6), 393 (30), 355 (8), 295 (7), 255 (7), 241 (9), 173 (12), 95 (45), 69 (100), 55 (51).
To ensure that \textit{in vivo} biosynthesized substrate did not contribute to the product profile, pLD2.5, the plasmid containing CAS1I481V, was transformed into LHY4.\textsuperscript{173,228} The procedure described above for SMY8[CAS1 I481V] was followed to analyze the enzymatic product profile. GC analysis of the acetylated products indicated a relative ratio of 54 \textit{cycloartenol}:25 \textit{lanosterol}:21 \textit{parkeol}. These values are essentially the same relative ratios obtained in the SMY8 host expression strain. SMY8[CAS1]\textsuperscript{204} was grown under inducing conditions, and enzymatic product was isolated in a similar manner as described above. The enzymatic products were determined to be 99 \% \textit{cycloartenol} and 1 \% \textit{parkeol} by GC analysis.

\textbf{Arabidopsis thaliana CAS1 I481A}

\textit{Isolation of Enzymatic Products from A. thaliana CAS1 I481A.} A saturated 50 mL induced culture of LHY2[CAS1 I481A]\textsuperscript{229,230} was transferred in equal volumes to two tared Falcon tubes. The cells were harvested by centrifugation (1300 \times g, 5 min), and the supernatant was decanted. The wet cell weight was determined (\textasciitilde 1.1 g), and 10 mL buffer (0.1 M NaPO4, pH 6.4) was added to yield a 40 \% (w/v) slurry. The cells were lysed by three passes through a French Press (20,000 psi). The resulting cellular debris was collected by centrifugation (1300 \times g, 5 min), and the supernatant was transferred to a clean Falcon tube. An additional 10 mL aliquot of buffer was added to adjust the solution to 20\% of the original cell weight; one milliliter of OS (20 \times stock as described in \textit{Small scale in vitro assay for triterpene alcohol production}, p. 109) was added to start the \textit{in vitro} reaction. Enzymatic production was allowed to proceed at ambient temperature
for 12-16 h. Forty milliliters ethanol was added to stop the reaction. Residual cellular
debris and precipitates were collected by centrifugation (1300 × g, 5 min), and the
supernatant was transferred to a clean round bottom flask. The pellet was rinsed once
with 5 mL ethanol, and this solvent was combined with the original supernatant. The
flask was concentrated to half the volume by rotary evaporation. Pellicular silica was
added (~ 1 g, 230-400 mesh) to adsorb the detergent, and the mixture was concentrated to
dryness.

The silica was transferred to a chromatograph column, and polar organic compounds
were eluted with diethyl ether. The ethereal eluent was dried over MgSO₄, filtered,
concentrated in vacuo (156 mg crude), then chromatographed (1:1 hexane/diethyl ether) to
afford 4 mg enzymatic product (>90 % pure). The enzymatic product was acetylated as
described in Methods (see p. 110), and the terpene acetates were analyzed by GC. The
product profile afforded achilleyl, camilleyl, lanosteryl, parkeyl, and cycloartenyl
acetates in relative ratios of 13:6:54:15:12, respectively.

**Arabidopsis thaliana CPII**

*Purification of Cycloeucalenol.* Purification of cycloeucalenol was performed by argentlic
HPLC. The free alcohol was injected on a semi-preparative silica column (Nucleosil
SA, 250 x 10 mm, 5 μm, 100 Å) that had been impregnated with silver (Ag⁺). Isocratic
elution using methyl tert-butyl ether (MTBE) mixed in a 1:2 ratio with hexane (Hex) as
mobile phase at a flow rate of 2 mL/min afforded ~90 % pure cycloeucalenol. The
structure of the triterpene alcohol was confirmed by ¹H-NMR, ¹³C-NMR, GC, and
GC/MS fragmentation compared to authentic standard (a generous gift from Prof. J.-L. Giner). Characteristic spectroscopic information obtained from purified cycloeucalenol includes the following:

$^1$H-NMR (500 MHz, CDCl$_3$, 25°C): $\delta$ 0.142 (d, 1H, J = 4 Hz), 0.387 (d, 1H, J = 4 Hz), 0.895, (s, 3H), 0.898 (d, 3H, J = 6.3 Hz), 0.972 (s, 3H), 0.975 (d, 3H, J = 6.1 Hz), 1.022 (d, 3H, J = 6.7 Hz), 1.036 (d, 3H, J = 6.7 Hz), 2.233 (septet of d, 1H, J = 6.7, 1.1), 3.215 (m, 1H, J = ~ 5 Hz), 4.666 (qt, 1H, J = 1.5), 4.716 (ddd, 1H, J = 0.6, 1.1, 1.8 Hz).

$^{13}$C-NMR (500 MHz, CDCl$_3$, 25°C) $\delta$ 14.37 (C-29), 17.77 (C-18), 18.32 (C-21), 19.11 (C-32), 21.85 (C-27), 21.98 (C-26), 23.53 (C-9), 24.65 (C-6), 25.15 (C-11, C16, or C7), 26.95 (C-11, C-16, or C-7), 27.23 (C-19), 28.09 (C-11, C-16, or C-7), 30.77 (C-1), 31.30 (C-23), 32.86 (C-12, C-15), 33.79 (C-25), 34.80 (C-2), 34.99 (C-22), 35.32 (C-12, C-15), 36.11 (C-20), 43.31 (C-5), 44.58 (C-4), 45.33 (C-13), 46.86 (C-8), 52.19 (C-17), 76.60 (C-3), 105.90 (C-28), 1576.9 (C-24). GC/MS: $m/z$ = 426 (51), 411 (37), 408 (67), 393 (40), 300 (30), 286 (5), 245 (11), 55 (100).

In vitro expression of pML1.8. E. coli expression and in vitro characterization of the putative A. thaliana CPI1 was performed as described by Rahier et al. The expression plasmid containing the putative cycloeucalenol cycloisomerase, pML1.8, was prepared by M. Lovato as described previously and transformed into BL21(DE3)pLysS (Novagen) as described in the Methods (see p. 36) section. One fresh transformant was inoculated into 5 mL LB media supplemented with carbenicillin (100 $\mu$g/mL) and
chloramphenicol (100 μg/mL) and allowed to grow overnight with aeration at 37 °C. The saturated culture was used (500 μL) to inoculate 25 mL LB containing 100 μg/mL carbenicillin and 25 μg/mL chloramphenicol, then allowed to grow at 37 °C. At the end of one hour, 250 μL 100 mM IPTG was added to induce expression of the AtCPI1 protein, and the culture was shaken for 5 h at 37 °C. The cells were collected by centrifugation (1300 × g, 15 min), rinsed with sterile deionized water, then resuspended to 20 % (w/v) in buffer (0.1 M Tris HCl, pH 7.8, 5 mM DTT, 4 mM MgCl₂, 0.2 % (v/v) Tween 80).

Purified cycloecualenal (see below) was added from an ethanolic solution to the induced bacterial cells to 100 μM. The cells were lysed by sonication (10 sec, 3 cycles) while being kept on ice then incubated overnight at ambient temperature. The reaction was quenched with 2 volumes of cold ethanol; denatured proteins were collected by centrifugation (1300 x g, 15 min). The ethanolic supernatant was transferred to a round bottom flask and concentrated by rotary evaporation. The residue was extracted thrice with diethyl ether, and the combined organic extracts were passed through a silica and MgSO₄ plug, then concentrated in vacuo. The reaction products were silylated as described in Methods (see p. 110). GC analysis confirmed the presence of a peak co-eluting with obtusifoliyl tetramethyisilyl ether. Characteristic spectroscopic information obtained from the obtusifoliol standard (a generous gift from Prof. J.-L. Giner) was used to confirm the formation of the Δ-8 triterpene alcohol as a result of the isomerization; these data are included below.

¹H-NMR (400 MHz, CDCl₃, 25°C): δ 0.712 (s, 3H), 0.888 (s, 3H), 0.929 (d, 3H, J =
6.3 Hz), 0.970 (s, 3H), 1.024 (d, 3H, J = 6.8 Hz), 1.029 (d, 3H, J = 6.8 Hz), 4.664 (s, broad, 1H), 4.717 (s, broad, 1H), 3.103 (m, 1H, J = ~ 5 Hz).

GC/MS: m/z = M⁺ (relative intensity ratio), 426 (78), 411 (87), 393 (12), 327 (18), 285 (10), 259 (13), 245 (34), 69 (100).
CHAPTER 8: RESULTS AND DISCUSSION

Characterization of Sterol Biosynthetic Enzymes

Sterol biosynthesis constitutes an intriguing metabolic pathway in which to analyze evolutionary bias. Subtle biochemical differences in common pathways can be observed among the eukaryotes. One of those common pathways is sterol biosynthesis, and the differential cyclization of the common precursor (S)-2,3-oxidosqualene represents one subtle biochemical difference that certainly attributes to the specialization of higher plants. The following discussion contemplates the enzymatic differences in plants relative to fungi and animals at the site of oxidosqualene cyclization.

Cyclization of Oxidosqualene in Plants versus Fungi

Directed evolution of *A. thaliana* cycloartenol synthase (CAS1) permitted the identification of an I481V mutation by screening for altered enzymatic specificity.\(^{173}\) The spontaneous mutation effected a change in the chemical transformation, specifically introducing a degree of freedom the distance of one carbon-carbon bond length (1.53 Å) into the active-site cavity. Relieving steric hindrance compromised product specificity, thereby allowing deprotonation from C-19 (cycloartenol), C-8 (lanosterol), and C-11 (parkeol) (Figure 11).\(^{173}\) Consequently, the deprotonation step yielded three, rather than one, triterpene alcohol products. The native Ile481 corresponds to Asp374 in squalene-hopene cyclase,\(^{236}\) which has been identified as an active-site residue.\(^{214}\) However, the hydrophobic sec-butyl side chain of isoleucine bears no obvious chemical resemblance to

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the charged polar side chain of aspartic acid. The I481V mutation represents the first incidence of purely hydrophobic effects controlling chemical catalysis. Furthermore, based solely on sequence alignments of OSCs and SHCs, the importance of I481 to OSC catalysis could not have been inferred from the corresponding SHC D374. Consequently, the identification of I481 as an active-site residue in OSCs is beyond the scope of DNA sequence analysis.

![Figure 11. Product profile of A. thaliana CAS1 I481V](image)

Further investigation of the CAS1 I481 residue indicated that by further decreasing the space in the active site occupied by this residue, steric hindrance was relieved, thereby leading to a compromised cyclization step. The I481A mutation afforded a suite of
triterpene alcohol products (Figure 12) resulting not only from variations in deprotonation mechanisms but also from alterations in cationic stabilization mechanisms. The formation of cycloartenol, lanosterol, and parkeol (all tetracycles) were expected based on the I481V results, but the unexpected formation of monocycles achilleol A \( ^{231} \) and camelliol \( ^{232} \) indicated that Ile481 was essential for maintaining spatial orientation of the substrate.\(^{229}\) The observed product profile suggests that the isoleucine residue (I481) affects not only the accuracy of deprotonation but also the formation of the A/B and B/C \textit{trans}-ring fusions. Intuitively, inefficient ring fusions can result from alterations in specific \(\pi\)-cation interactions caused by the mutated residue; these interactions are believed to stabilize the intermediate.

Figure 12. Product profile of \textit{A. thaliana} CAS1 I481A

\(122\)
cational structures. Given that Ile481 is electronically neutral, a disrupted stabilization network cannot explain how mutations at this residue effect a decrease in catalytic specificity. More likely, the steric effects controlling catalysis in the active-site cavity of the enzyme (Cas1p) have been altered as a result of a subtle change in the structure of the cavity.

The small change in the active site induced by replacing isoleucine with alanine (CAS1 I481A) caused inefficient synthesis of cycloartenol (12% relative ratio compared to 99% from CAS1). Despite the severe changes in the product profile of CAS1 I481A, the mutant protein retained its original 38% sequence identity to S. cerevisiae ERG7 (which cyclizes oxidosqualene solely to lanosterol\textsuperscript{173}).\textsuperscript{229} This point mutation readily altered the active site permitting biosynthesis of isomers including the sterol precursor lanosterol. Despite the vulnerability exhibited by Cas1p in directing cycloartenol formation \textit{in vitro}, plants maintain a \textit{de novo} biosynthesis of cyclopropyl sterols in Nature.

Similarly, derivation of the I481V mutation in cycloartenol synthase required a mere 17 days of cultivation under sterol-deprived conditions. Consequently, this mutation would arise frequently in Nature unless a constant selective pressure was maintained to prevent the evolution of lanosterol and/or parkeol formation. Perhaps structural accuracy may not be critical for the physiological utility imparted by cyclopropyl sterols. Precedence for structural diversity in terpene production has been observed in conifers with respect wound response.\textsuperscript{12}

The observed vulnerability of CAS1 product specificity is not shared by the yeast
analog ERG7; similar studies to alter the catalytic specificity in S. cerevisiae ERG7 emphasized that the formation of lanosterol cannot be so easily manipulated.228

Most of the higher plants investigated utilized the tandem pair of cycloartenol synthase-cyclopropyl isomerase *en route* to sterols, which serve the same bulk membrane requirement in plant cells that sterols serve in fungi.237 Only one example has been documented that describes the formation of lanosterol from oxidosqualene in a higher plant.238 Because the physiological relevance of cyclopropyl sterols in higher plants continues to elude scientists, the reason plants evolved a catalytically different OSC also remains unknown.

Generally, natural selection favors the evolution of strategies that increase the rate an organism adapts to its environment. Because higher plants evolved not only the unique OSC activity to produce pentaacyclic sterols, but also the ability to metabolize them to tetracyclic sterols, a physiological purpose must be met by the accumulation of cyclopropyl sterols. Otherwise, molecular economy dictates that the route used by animals and fungi is more efficient. To investigate the unique cyclopropyl isomerase activity observed in higher plants, the functional characterization of the *A. thaliana* cyclopropyl isomerase gene (CPI1) is described.

*A. thaliana* Cycloecualenol Cycloisomerase

Previous attempts to characterize the putative *A. thaliana* CPI1 gene produced inconclusive results because of an impure substrate.239 The substrate previously used contained several compounds that were identified by 1H-NMR to be structural isomers of
cycloeucalenol. Because these contaminating isomers differed primarily in the double bond location on the sterol side-chain, argentie HPLC\(^{233}\) successfully purified the major product, cycloeucalenol, to ~ 90% (by GC). This approach allowed the separation of isomers by simultaneously exploiting polarity and π-bond character, thereby, permitting functional characterization of the first cycloeucalenol cycloisomerase (CPI1).

The chemical transformation catalyzed by CPI1 is illustrated in Figure 13. During catalysis, a presumed water molecule donates a proton to C-19,\(^{218}\)

![Chemical structure of cycloeucalenol and obtusifoliol](image)

Figure 13. Reaction catalyzed by *A. thaliana* cycloeucalenol cycloisomerase

which opens the cyclopropyl ring and leaves a carbonium ion at C-9. The CPI1 protein (Cpi1p) then abstracts a proton from C-8\(^{240}\) to quench the cation at C-9. As a result, a tetrasubstituted double bond replaces the three-membered ring; the formation of obtusifoliol represents the plant Δ8-sterol analog to lanosterol (Figure 13). However, unlike biosynthetic lanosterol found in fungi and animals, the obtusifoliol structure shows evidence of metabolism, namely the demethylation of the 4β-methyl and the introduction of the Δ-24(28) bond on the side chain. These processes must occur prior to cyclopropyl isomerization to obtain optimal catalytic activity.\(^{217,241}\) Consequently, the
preferred substrate of cyclopropyl isomerase enzymes is cycloeucalenol rather than cycloartenol.

Yeast sterol biosynthetic enzymes responsible for the metabolism of lanosterol to ergosterol possess relatively broad substrate specificity\textsuperscript{242} which allowed for the successful cloning of the \textit{A. thaliana} CPI1.\textsuperscript{235} A yeast strain was engineered to emulate a plant mutant in cyclopropyl isomerase activity (\textit{cpf}), but rather than accumulating cycloeucalenol, cycloartenol served as the representative cyclopropyl sterol. Cycloartenol retains both 4α and 4β methyl groups and, therefore, should have been a very poor substrate for CPI1.\textsuperscript{217,241} The engineered yeast were apparently able to partially demethylate cycloartenol which led to the formation of a more suitable substrate for the isomerase enzyme. Isomerization of the cyclopropyl ring to tetracyclic sterol that ultimately served to fulfill ergosterol requirement permitted viability in the strain containing the isomerase enzyme. The ability of yeast to compensate for a change in the biochemical characteristics of the essential sterol biosynthetic pathway, as demonstrated by this engineered yeast strain, confirms that survival remains the foundation for evolution. With the information described in this chapter, revealing the physiological importance of cyclopropyl sterols to plants will be facilitated.
CHAPTER 9: CONCLUSION

The use of molecular phylogeny allows insight into natural adaptation by highlighting genomic information that possibly contributed to an organism's growth advantage. Using directed evolution to initiate studies on a unique activity observed in plants, identification of putative catalytic residues contributing to the lanosterol-cycloartenol bifurcation resulted. Cycloartenol production in higher plants proved to be highly vulnerable to diminished cycloartenol biosynthesis because of alterations in the protein. Despite the vulnerability, plants have retained the ability to biosynthesize cyclopropyl sterols for a length sufficient to allow evolution of cyclopropyl isomerase activity to further metabolize the sterols. Why plants evolved the unique ability to biosynthesize cyclopropyl sterols cannot be explained to date. However, adding new data to molecular phylogenetic analysis allowed this thesis work to begin to answer how, and possibly why, plants evolved the means to biosynthesize cyclopropyl sterols.
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**APPENDIX I: RELEVANT S. CEREVISIAE ENZYMES**

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<thead>
<tr>
<th>Gene</th>
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APPENDIX II: LIST OF RELEVANT S. CEREVISIAE STRAINS

**Part I**

**JBY575**  
Wild type yeast  
\( \text{MAT}^a \text{ura3-52 trp1-}\Delta63 \text{leu2-3,112 his3-}\Delta200 \text{ade2 Gal}^+ \)

**EHY1**  
GGPP synthase under control of the GAL1 promoter  
\( \text{MAT}^a \text{leu2::LEU2::pGAL1-BTS1 ura3-52 trp1-}\Delta63 \text{his3-}\Delta200 \text{ade2 Gal}^+ \)

**LHY1**  
Heme auxotroph, squalene synthase deletion  
\( \text{MAT}^a \text{erg9::HIS3 hem1::TRP1 ura3-52 trp1-}\Delta63 \text{leu2-3,112 his3-}\Delta200 \text{ade2 Gal}^+ \)

**EHY9**  
Heme auxotroph, squalene synthase under control of CUP1 inducible promoter  
\( \text{MAT}^a \text{pCUP1-ERG9 hem1::TRP1 ura3-52 trp1-}\Delta63 \text{leu2-3,112 his3-}\Delta200 \text{ade2 Gal}^+ \)

**EHY10**  
Squalene synthase under control of CUP1 inducible promoter  
\( \text{MAT}^a \text{pCUP1-ERG9 ura3-52 trp1-}\Delta63 \text{leu2-3,112 his3-}\Delta200 \text{ade2 Gal}^+ \)

**EHY13**  
Squalene synthase under control of the CUP1 promoter, truncated HMG1 under control of the GAL1 promoter  
\( \text{MAT}^a \text{pCUP1-ERG9 pGAL1-trHMG1::LEU2 ura3-52 leu2,3-112 trp1-}\Delta63 \text{his3-}\Delta200 \text{ade2 Gal}^+ \)

**upc2-1**

**EHY18**  
upc2-1 incorporated, truncated HMG1 under control of GAL1 promoter, GGPP synthase under control of the GAL1 promoter  
\( \text{MAT}^a \text{pGAL1-BTS1::his pGAL1-trHMG1::LEU2 upc2-1 ura3-52 trp1-}\Delta63 \text{leu2-3,112 his3-}\Delta200 \text{ade2 Gal}^+ \)

**EHY19**  
upc2-1 incorporated, truncated HMG1 under control of the GAL1 promoter, GGPP synthase under control of the GAL1 promoter  
\( \text{MAT}^a \text{pGAL1-BTS1::hisG pGAL1-trHMG1::LEU2 upc2-1 ura3-52 leu2,3-112 trp1-}\Delta63 \text{his3-}\Delta200 \text{ade2 Gal}^+ \).

**EHY24**  
FPF synthase under control of the GAL1 promoter  
\( \text{MAT}^a \text{pGAL1-ERG20::LEU2 upc2-1 ura3-52 leu2,3-112 trp1-}\Delta63 \text{his3-}\Delta200 \text{ade2 Gal}^+ \)
erg9-1  

EHY315  
upc2-1 and erg9-1  
(MATα upc2-1 erg9-1 ura3-52 leu2-3,112 his3–Δ200 ade2 Gal+)  

EHY31  
truncated HMG1 under control of the GAL1 promoter, upc2-1, erg9-1  
(MATα pGAL1-trHMG1::LEU2 upc2-1 erg9-1 ura3-52 leu2,3-112 trp1-Δ63 his3–Δ200 ade2 Gal+)  

EHY32  
GGPP synthase under control of the GAL1 promoter, upc2-1, erg9-1  
(MATα pGAL1-BTS1::LEU2 upc2-1 erg9-1 ura3-52 leu2,3-112 his3–Δ200 ade2 Gal+)  

EHY33  
FPP synthase under control of the GAL1 promoter, upc2-1, erg9-1  
(MATα pGAL1-ERG20::LEU2 upc2-1 erg9-1 ura3-52 leu2,3-112 his3–Δ200 ade2 Gal+)  

EHY34  
FPP synthase under control of the GAL1 promoter, upc2-1, erg9-1  
(MATα pGAL1-ERG20::LEU2 upc2-1 erg9-1 ura3-52 leu2,3-112 trp1-Δ63 his3–Δ200 ade2 Gal+)  

EHY35  
upc2-1, erg9-1  
(MATα upc2-1 erg9-1 ura3-52 leu2,3-112 his3–Δ200 ade2 Gal+)  

EHY36  
GGPP synthase under control of the GAL1 promoter, upc2-1, erg9-1  
(MATα pGAL1-BTS1::LEU2 erg9-1 ura3-52 leu2,3-112 his3–Δ200 ade2 Gal+)  

EHY37  
erg9-1  
(MATα erg9-1 ura3-52 ade2 Gal+)  

EHY40  
erg9-1  
(MATα erg9-1 ura3-52 leu2-3,112 his3–Δ200 ade2 Gal+)  

Part II  

SMY8  
heme auxotroph, lanosterol synthase deletion  
(MATα erg7::HIS3 hem1::TRP1 ura3-52 trp1–Δ63 leu2-3,112 his3–Δ200 ade2 Gal+)  

LHY4  
heme auxotroph, lanosterol synthase deletion, squalene synthase deletion  
(MATα erg9::HIS3 erg7::hisG hem1::TRP1 ura3-52 trp1–Δ63 leu2-3,112 his3–Δ200 ade2 Gal+)  

MLY1  
cyclooleucalenol cycloisomerase under control of the GAL1 promoter, heme 

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auxotroph, lanosterol synthase deletion

(MATa pGAL1-CPI1::LEU2 erg7::HIS3 hem1::TRP1 ura3-52 trp1-Δ63 leu2-3,112 his3-Δ200 ade2 Gal"
APPENDIX III: LIST OF RELEVANT PLASMIDS

Part I

pEH1.3  
*S. cerevisiae* BTS1 subcloned at SalI/NotI into pRS305Gal

pEH1.4  
*S. cerevisiae* BTS1 subcloned at SalI/NotI into pRS426Gal

pEH9.0  
*A. grandis* abietadiene synthase subcloned at BamHI/NotI into pRS305Gal

pEH12.1  
*S. cerevisiae* truncated HMG1 subcloned at SalI/NotI into pRS305Gal

pEH12.2  
*S. cerevisiae* truncated HMG1 subcloned at SalI/NotI into pRS314Gal

pLH4.12  
*A. annua* epicedrol synthase subcloned at SalI/NotI into pRS426Gal

pJR9.2  
*F. sporotrichioides* trichodiene synthase subcloned at NdeI/NotI into pRS426Gal

pNKY85  
*S. cerevisiae* URA3 flanked by bacterial repeats (hisG) (see ref. 170)

Part II

pLD2.5  
*A. thaliana* CAS1 I481V subcloned at SalI/NotI into pRS426Gal

pSM60.22  
*A. thaliana* CAS1 subcloned at SalI/NotI into pRS426Gal

pML1.8  
*A. thaliana* CPI1 subcloned at NdeI/NotI into pET-15b