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(54) **METHOD AND SYSTEM FOR DITERPENE PRODUCTION PLATFORMS IN YEAST**

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C12N 15/81 (2006.01)
C12N 15/01 (2006.01)

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CPC **C12N 15/81** (2013.01); **C12N 15/01** (2013.01)

(58) **Field of Classification Search**
None
See application file for complete search history.

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(57) **ABSTRACT**

A method is provided for modifying yeast to express mutant avian farnesyl diphosphate synthase and the resulting modified yeast. The yeast advantageously includes additional mutants including but not limited to having ergosterol dependent growth and being erg-. The modified yeast are beneficial for the production of various terpenes including diterpenes.

16 Claims, 15 Drawing Sheets

Specification includes a Sequence Listing.

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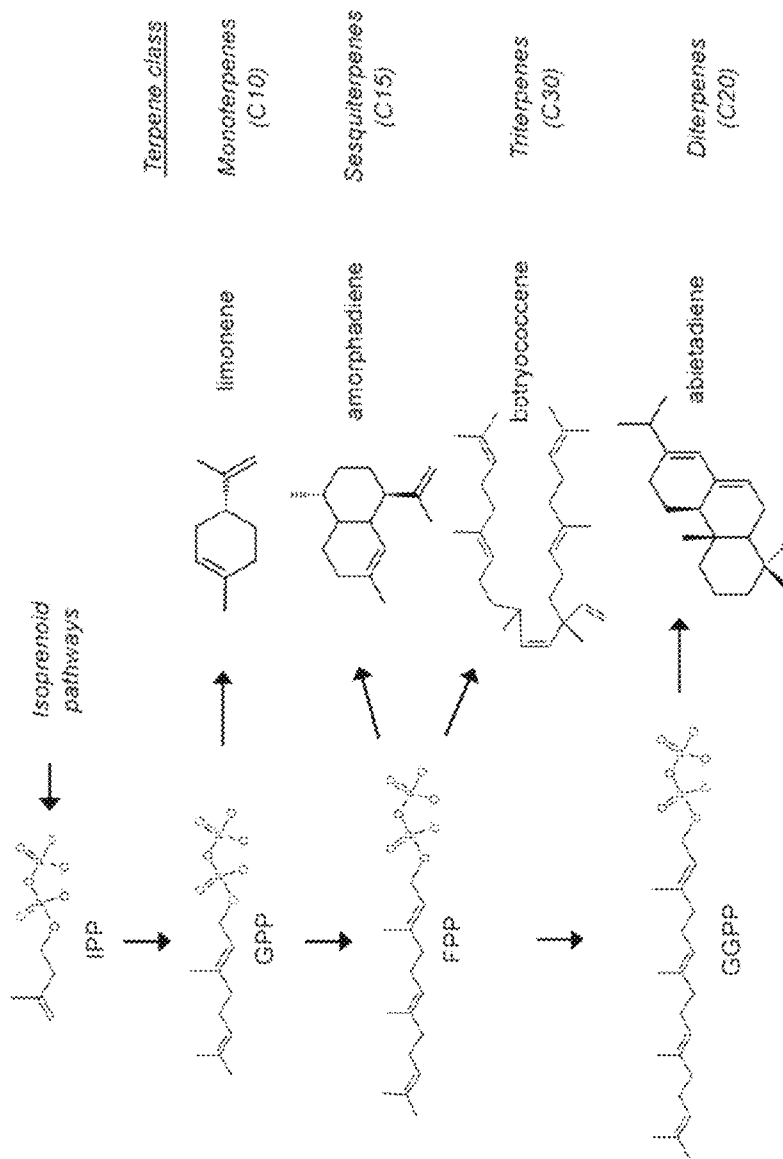


Figure 1

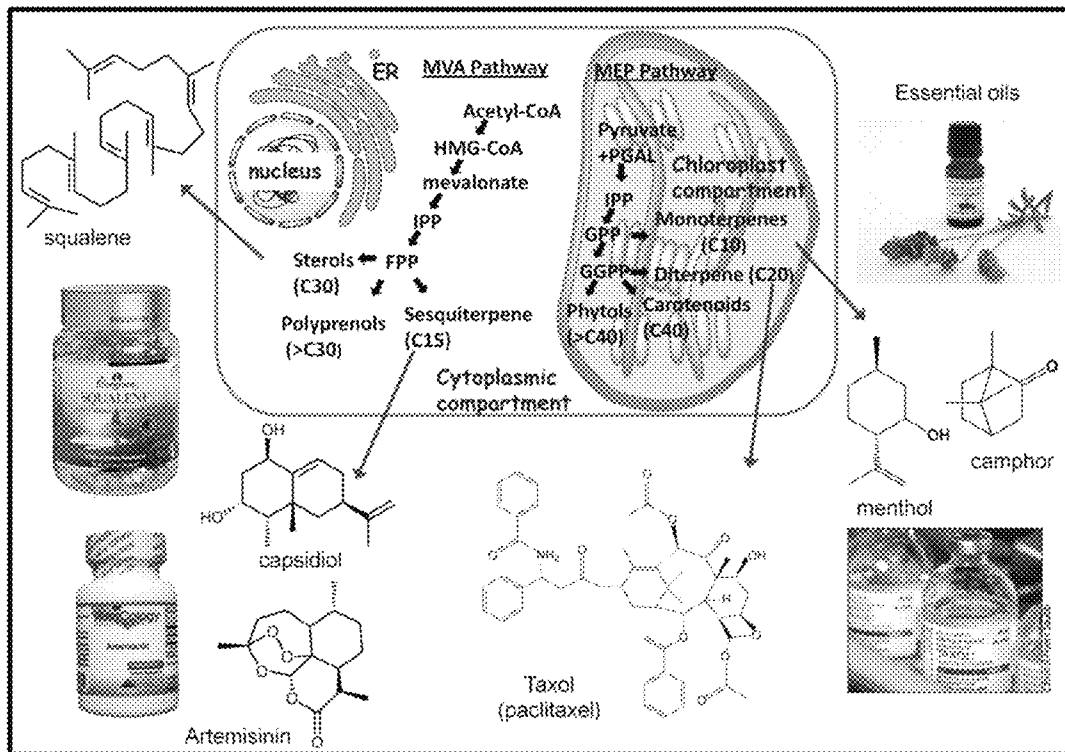


Figure 2

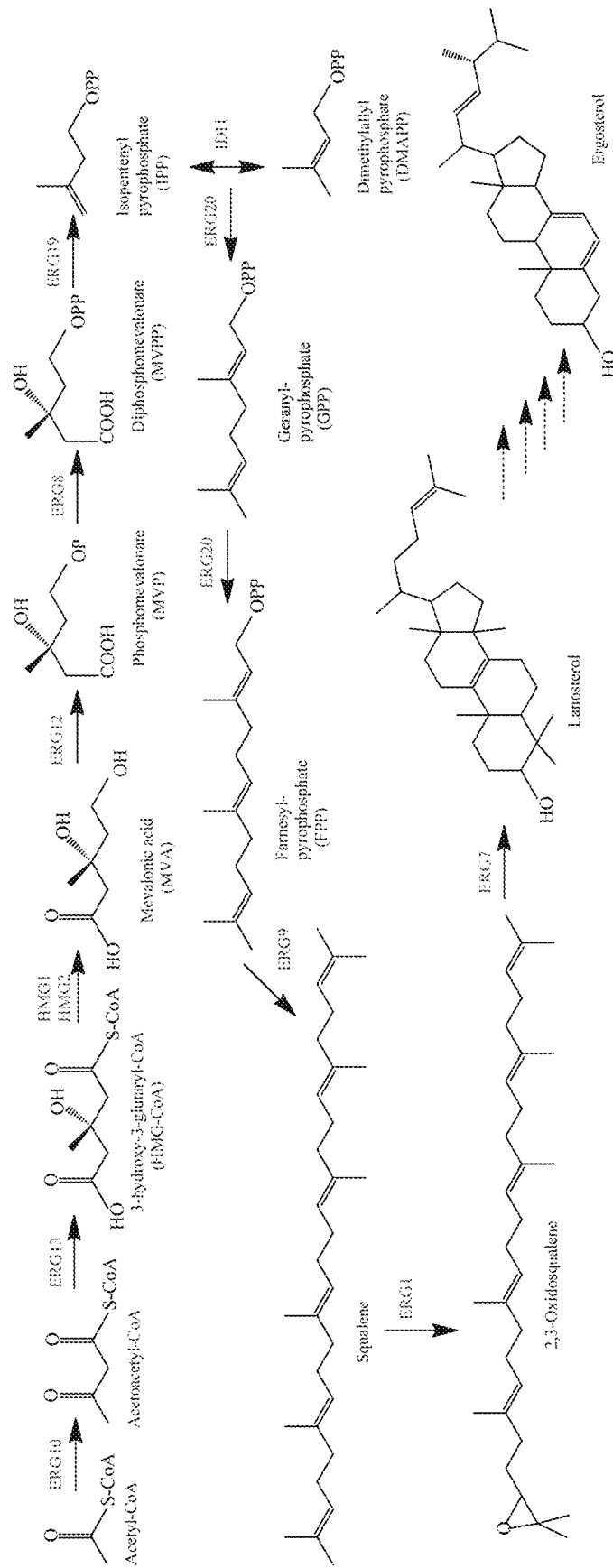


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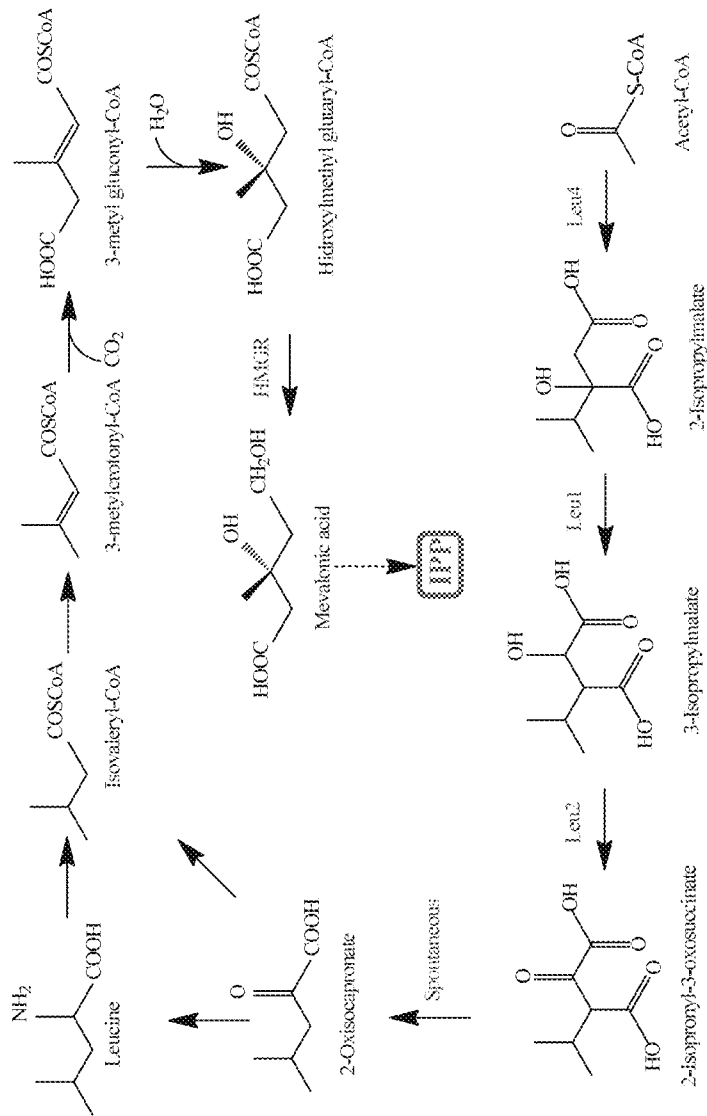


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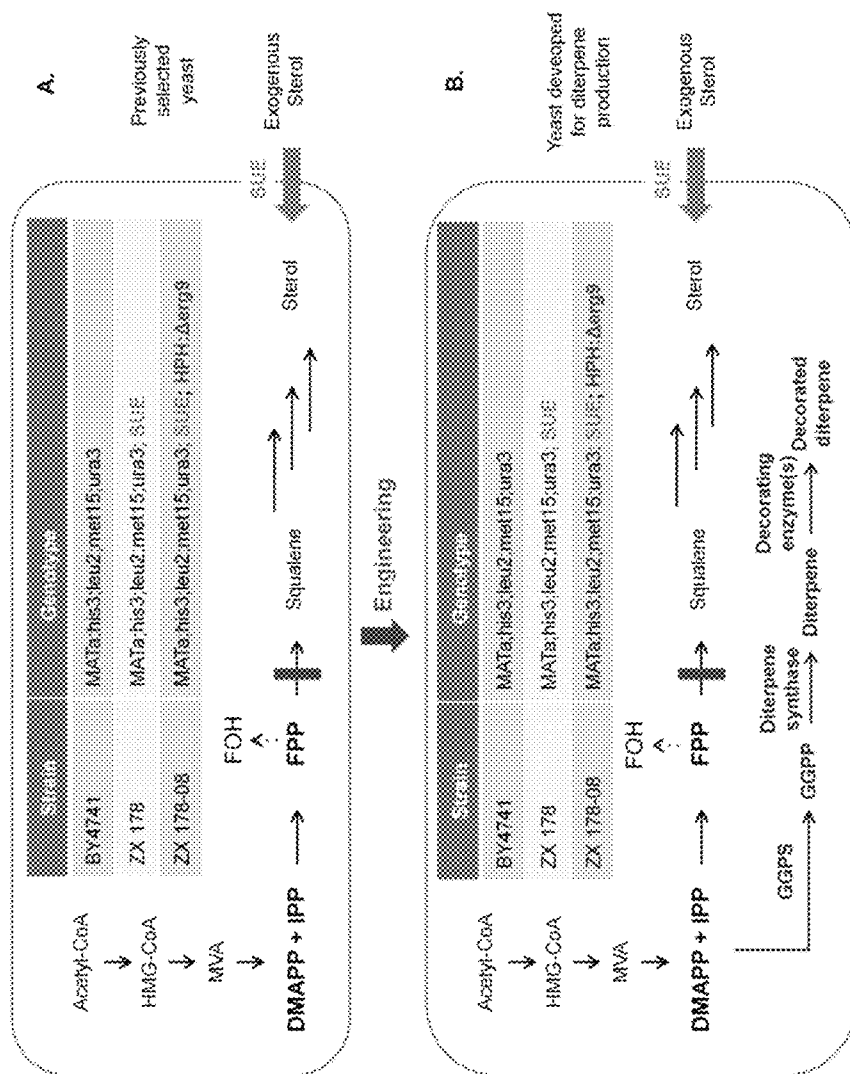


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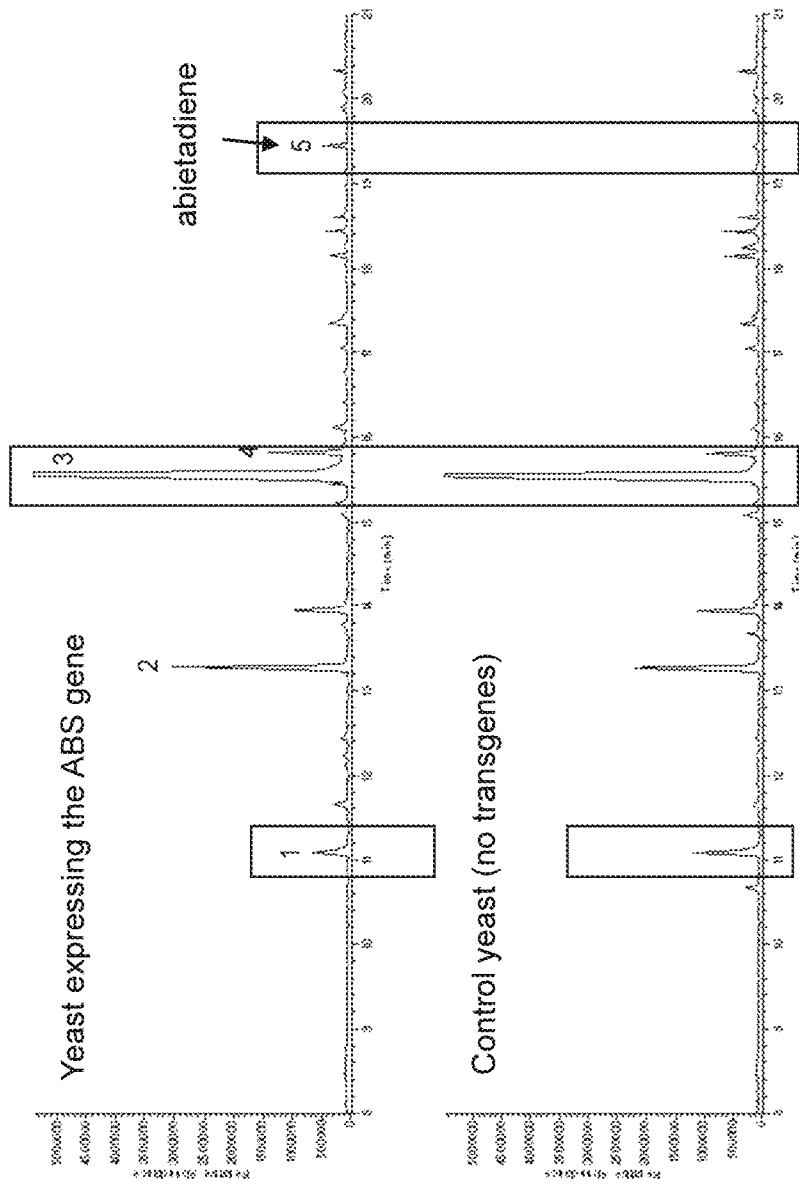


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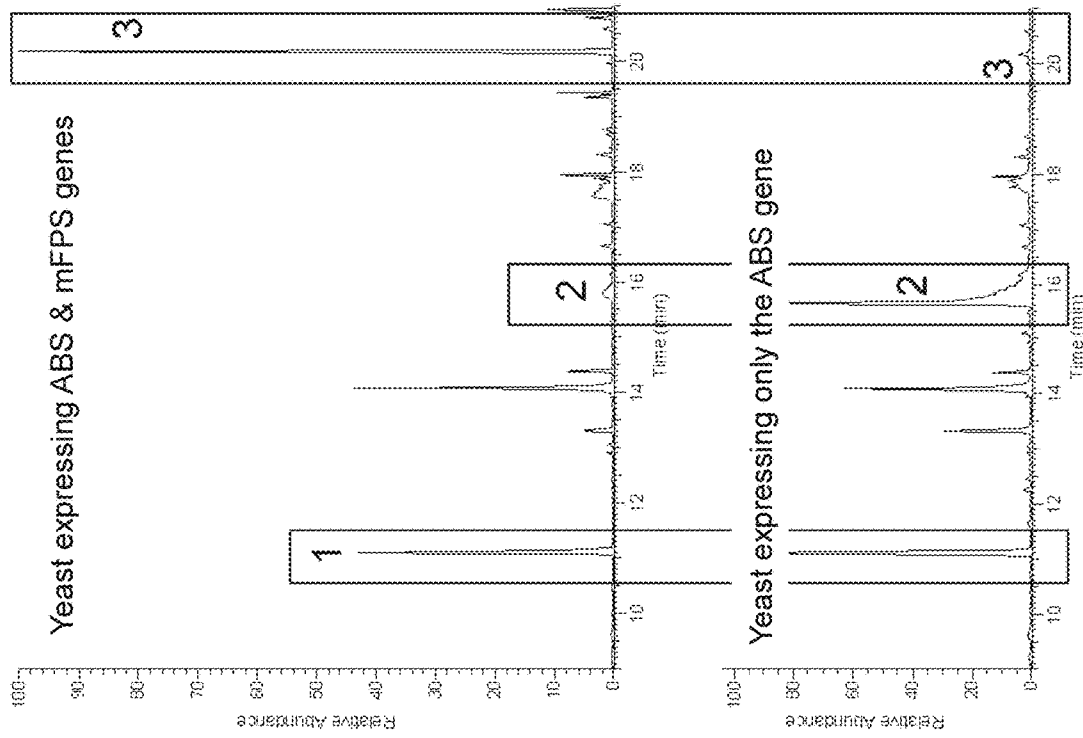


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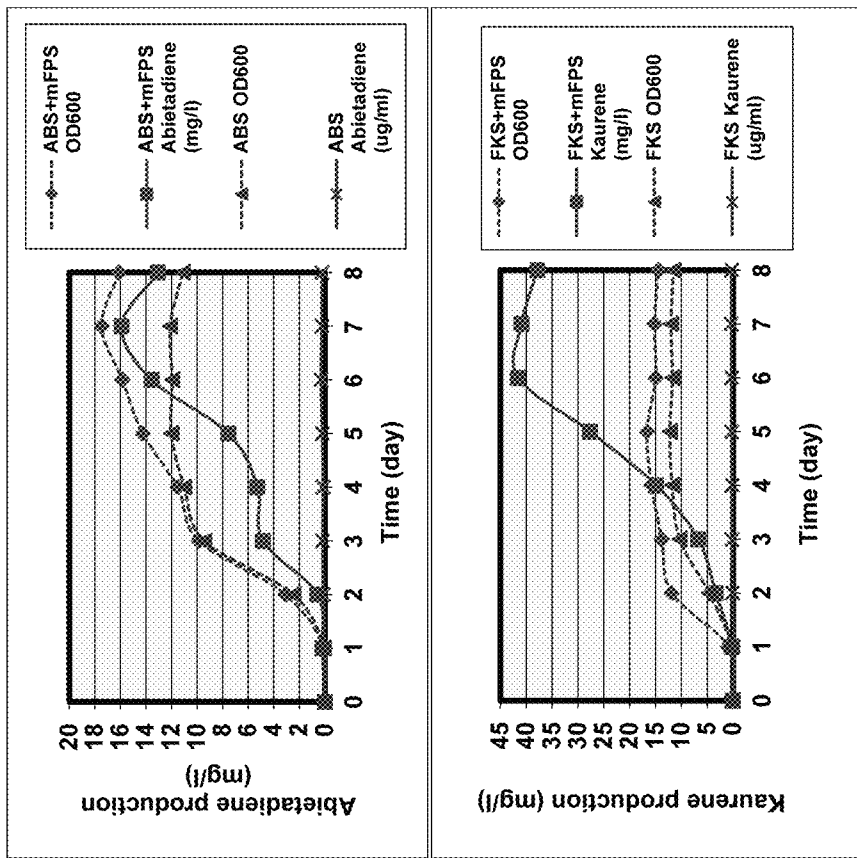


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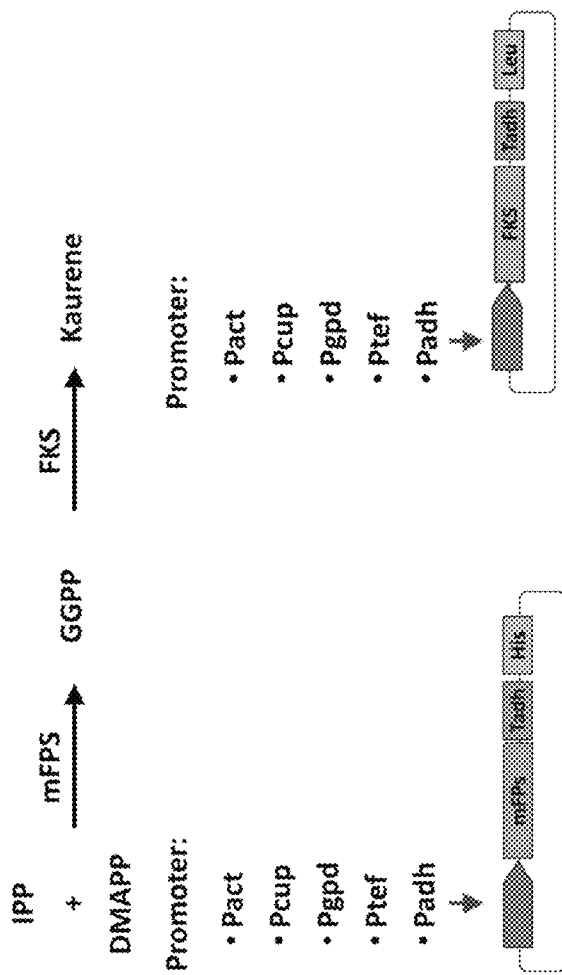


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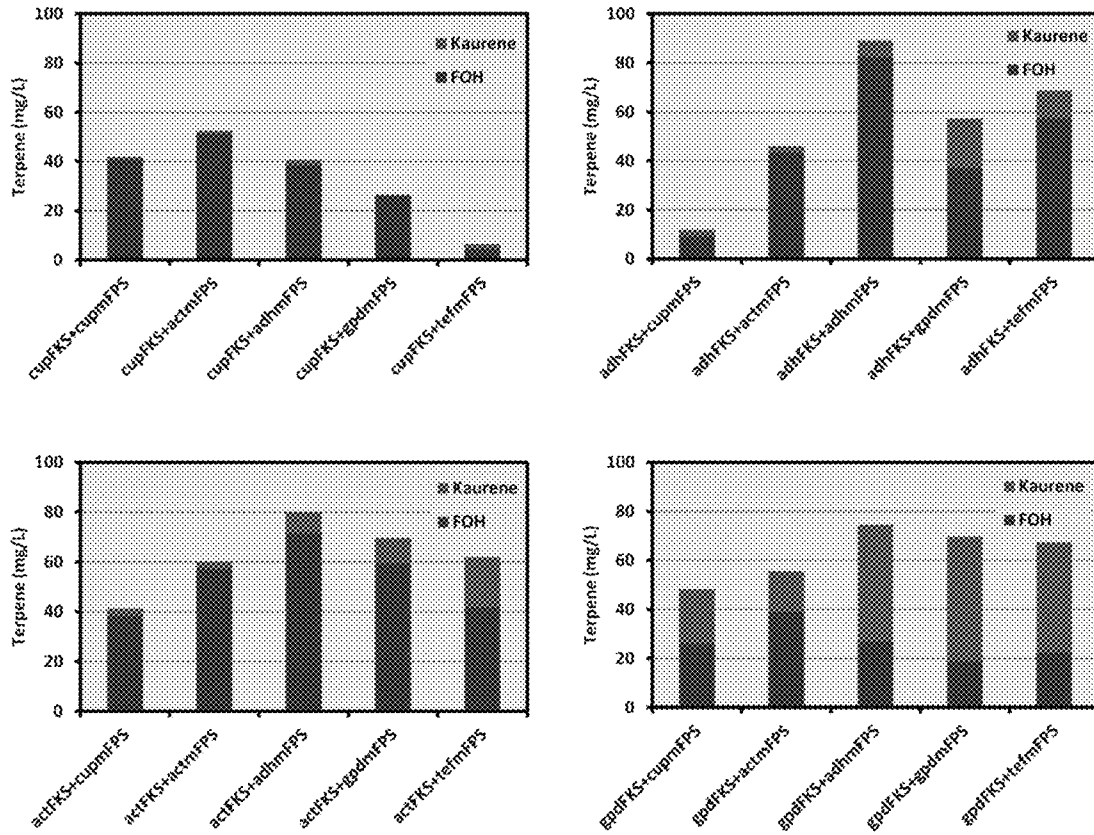


Figure 10

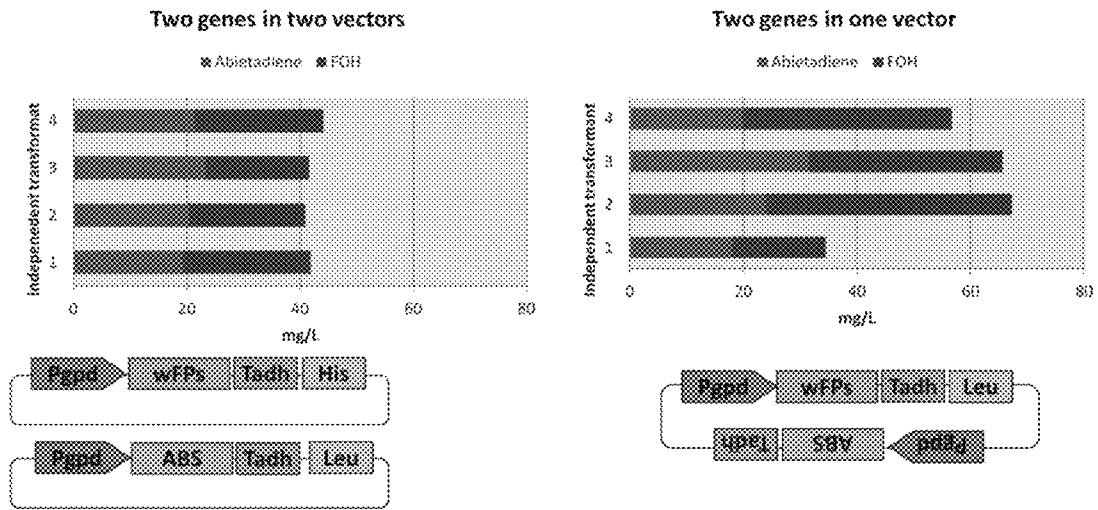


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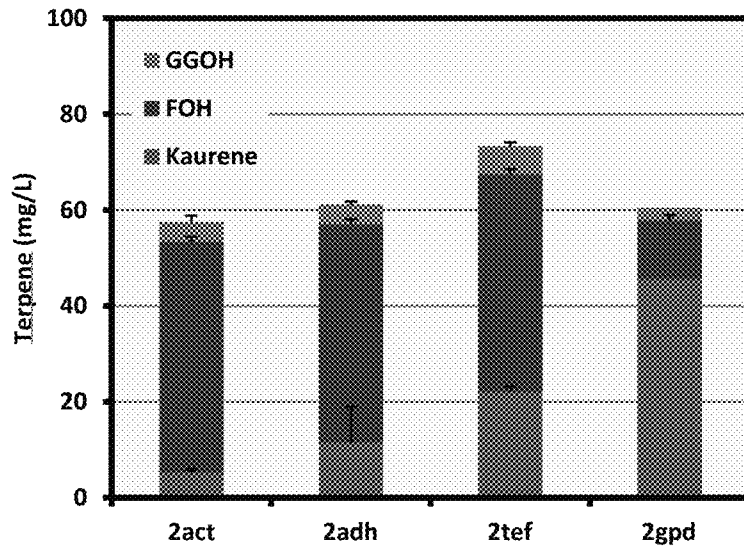


Figure 12

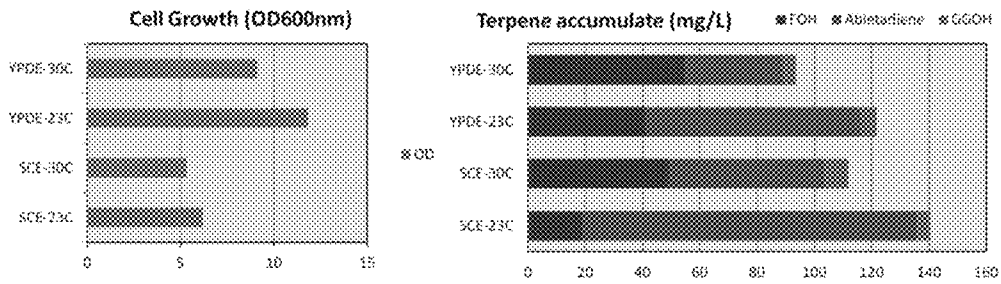


Figure 13

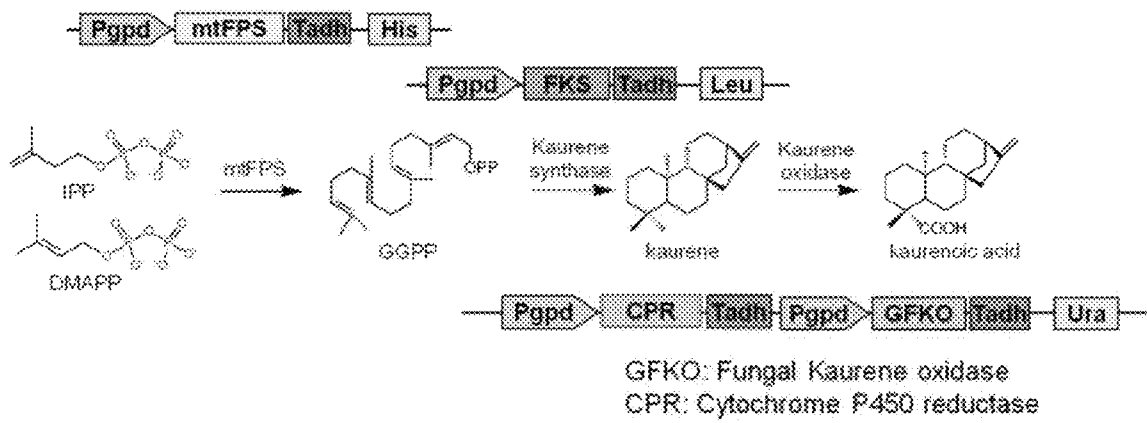


Figure 14

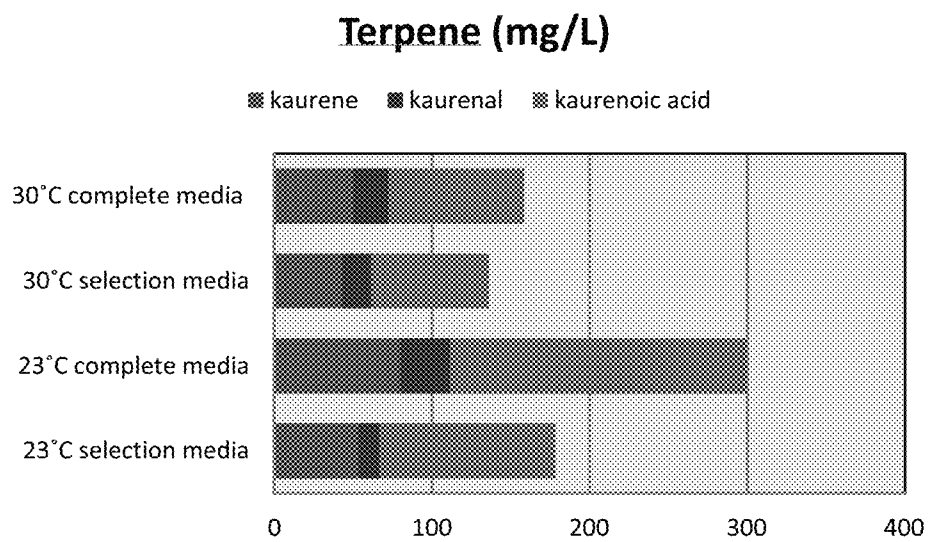


Figure 15

METHOD AND SYSTEM FOR DITERPENE PRODUCTION PLATFORMS IN YEAST

CROSS-REFERENCE TO RELATED APPLICATION

This application claims the benefit of U.S. provisional patent application Ser. No. 61/730,412, filed Nov. 27, 2012, and co-pending application filed Nov. 27, 2013, both herein incorporated by reference.

FIELD OF THE INVENTION

The presently-disclosed subject matter relates to methods for producing or generating modified yeast, and the resulting yeast, and in particular, yeast that can be used for various aspects of terpene production. For example, the subject matter relates to methods and systems for building terpene production platforms in yeast to express mutant avian farnesyl diphosphate synthase. These platforms or cell lines can be further modified, e.g. genetically engineered to produce specific enzymes and/or terpenes, namely diterpenes.

BACKGROUND OF THE INVENTION

Plants, microorganisms and animals produce a large variety of organic chemical compounds, some of which are used universally for growth and metabolism and others seem to play specialized roles in the life cycle of the organism (Maimone & Baran, 2007). As such, two large classes of natural products are widely recognized. Primary metabolites are those essential for life in all eukaryotic organisms, while specialized metabolites appear to give species specific advantages for occupying distinct environmental niches. The distinctive role specialized metabolites play in an organisms natural history, for example how these metabolites provide protection against microbial challenge, have also not escape attention for their possible utility in a wide range of applications. For example, many of the currently used drugs are derived or inspired from plant-derived specialized chemicals and are commonly referred to as Natural Products (Buchanan et al., 2002). Capturing the chemical and structural diversity of Natural Products has recently been identified as a major objective within the scientific community in large part because of the wide array of applications Natural Products can have and the resulting economical implications.

Terpenes and terpenoids are a large and diverse family of Natural Products with more than 55,000 having been identified (Maimone & Baran, 2007). However, based on the biosynthetic mechanisms responsible for terpenes, chemists have predicted that only a small fraction of all the possible terpene compounds have been discovered (Bouvier et al., 2005). Terpenes are derived from the five carbon isoprene unit with different combinations of the isoprene units generating different classes of the terpene products. The classification and biosynthesis of terpenoids are based on the number of five-carbon units they contain as illustrated in FIG. 1. Monoterpenes (consisting of 10 carbons), sesquiterpenes (15 carbon derivatives), and diterpenes (20 carbon derivatives), arise from the corresponding intermediates geranyl diphosphate (GPP), farnesyl diphosphate (FPP), and geranylgeranyl diphosphate (GGPP). These intermediates in turn arise by the sequential head to tail condensation of C5 units. Higher order terpenes like triterpene (30 carbons) are formed from two farnesyl units condensed head-to-head.

Likewise, tetraterpenes (40 carbons) are formed from two geranylgeranyl units condensed head-to-head.

Monoterpenes are well known as the volatile essence of flowers and plants and such mixtures can account for up to 5% of plant dry weight (Buchanan et al., 2002). Menthol and camphor are common monoterpenes found in diverse plant families and whose structural complexity in terms of stereo- and regio-chemistry are emphasized in FIG. 2. Besides providing pleasing fragrances, monoterpenes have been shown to function as signal molecules in defense mechanisms against pathogens (Hick et al., 1999). Hence, monoterpenes have the commercial value as flavors, fragrances, essential oils, and as anticancer and antimicrobial drugs (Burke et al., 1997). Sesquiterpenes (C15) are also found in essential oils, and many sesquiterpenes possess antibiotic activities, prompting suggestions that they are produced by plants as a defense mechanism. Diterpenes (C20) include gibberellins (plant hormones), vitamin A, as well as pharmaceutical important metabolites such as taxol, an exceptional anticancer reagent (Barkovich & Liao, 2001). Triterpenes (C30) include the brassinosteroids, phytosterols important for lipid membrane composition, and components of surface waxes, such as oleanolic acid of grapes. Squalene, the major content of shark liver oil, is a linear triterpene and common ingredient in cosmetic products (Buchanan et al., 2002), has special utility as a lubricant for high performance machinery, and is a common adjuvant in many pharmaceutical formulations (Bhilwade et al., 2010, Huang et al., 2009, Reddy & Couvreur, 2009). Tetraterpenes (C40) include carotenoid accessory pigments, like lycopene, the monocyclic gamma-carotene, and the bicyclic alpha- and beta-carotenes, which perform essential for the light reactions of photosynthesis. Longer chain terpenes, so-called polyterpenes, contain more than 8 isoprene units and include examples like ubiquinone and rubber (Buchanan et al., 2002).

There are two pathways for terpene biosynthesis in plant cells. One is the mevalonate pathway (MVA) which is well established and discovered in the 1960s (Bouvier et al., 2005). The other is the mevalonate independent pathway, or more properly referred to as the methylerythritol-phosphate pathway (MEP), which was more recently discovered (Bouvier et al., 2005). The MEP pathway was first discovered in prokaryote cells, and then confirmed to exist in plant cells (Barkovich & Liao, 2001). Interestingly, plants utilize these two pathways to meet different terpene biosynthetic needs. Sesquiterpenes, sterols, triterpenes and oligoterpenes (side chain of dolichols) are synthesized in the cytosol via the MVA pathway, while monoterpenes, diterpenes, teraterpenes, and polyterpenoids are synthesized in chloroplasts via the MEP pathway using pyruvate and glyceraldehydes-3-phosphate as the primary precursors (FIG. 2).

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

lyzed by HMG-CoA synthase encoded by ERG13 to yield 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA).

TABLE 1

Biological activities and commercial applications of typical terpenoids			
Class	Biologic activities	Commercial applications	Examples
Monoterpenoids	Signal molecules and used as defense mechanisms against pathogens	Flavors, fragrances, cleaning products, anticancer, antibacterial, antioxidant, essential oil, bionief	Limonence, menthol, camphor, linalool
Sesquiterpenoids	Antibiotic, antitumor, antiviral, immunosuppressive, and hormonal activities, defensive agents or pheromones	Flavors, fragrances, pharmaceuticals (antibacterial, antifungal), insecticides, biofuels	Nootkatone, artemisinin, patchoulol, nerolidol, farnesol, capsidol, farnesene, bisabolene
Diterpenoids	Hormonal activities, growth regulator, antitumor, antimicrobial and anti-inflammatory properties	Anticancer agents, feedstock for industrial chemical applications	Gibberellins, phytol, taxot, kaurerte, abietadiene, kaurenoic acid, abietic acid
Triterpenoids	Membrane component, steroid hormones	Biologic markers, biofuel, skin moisturizers in cosmetics, immunologic adjuvant in vaccines.	Sterols, hopanoids, squalene, botryococcene.
Tetraterpenoids	Antioxidants, photosynthetic components, pigments, and nutritional elements (vitamins)	Food additives, colorants, antioxidants	Lycopene, beta-carotene

HMG-CoA is reduced by HMG-CoA reductase to yield mevalonate. This reaction is catalyzed by HMG-CoA reductase, which is encoded by 2 separate loci in yeast. Both loci appear to compensate for a knockout loss of the other gene. The C5 position of mevalonate is phosphorylated by mevalonate kinase, encoded by ERG12. Then a second kinase, phosphomevalonate kinase, encoded by ERG5, catalyzes the successive phosphorylation to yield diphosphomevalonate. In the next step the diphosphomevalonate is converted into IPP (isopentenyl diphosphate) by mevalonate diphosphate decarboxylase, encoded by ERG19. IPP isomerase, encoded by IDI1 converts IPP into DMAPP (dimethylallyl diphosphate). The condensation of the C5 building blocks of IPP and DMAPP into FPP is catalyzed by FPP synthase, which is encoded by ERG20. FPP can then be used as substrate for sterol and other isoprenoid biosynthetic needs.

Recent studies have discovered that FPP is also available in yeast mitochondria, as evidenced by increasing novel sesquiterpene production three-times by targeting a sesquiterpene synthase to the mitochondria compartment compared with targeting this same enzyme to the cytosol (Farhi et al., 2011). The origin of FPP in mitochondria could be the IPP and DMAPP arising in cytosol being imported and converted in the mitochondria to FPP. Alternatively, a hypothetical leucine metabolism model for the formation of terpene in *S. cerevisiae* is also a possibility. The leucine catabolism pathway (MCC pathway) is known to occur in the mitochondria of other eukaryotic mammal and plant

cells (Anderson et al., 1998), in mitochondria leucine metabolite to form 3-Hydroxy-3-methylglutaryl-CoA, which can be catalyzed by HMGR to produce mevalonic acid, and then produce IPP and DMAPP through MVA pathway as shown in FIG. 4 (Carrau et al., 2005). Interestingly, a yeast line engineered with a chimeric diterpene synthase targeted to the cytoplasm along with prenyltransferases streamlined for GGPP biosynthesis, yielded 2-3 times more diterpene when the expression vector also provided a leu2 auxotrophic selection marker gene. The interpretation provided by the authors was that the extra leucine produced by the auxotrophic selection marker gene provided another source for IPP via the leucine catabolic pathway (FIG. 4). (Zhou et al., 2012).

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

Terpenes are a very large class of structurally diverse compounds made by organisms in all kingdoms of life. The terpenes from plants are perhaps the most extensively described as evident by well over 100,000 different terpenes reported in the literature (Buckingham, 2003). Terpenes are also widely recognized for their diverse utility and applications. For example, taxol, a diterpene widely recognized for its application as a chemotherapeutic agent, was first isolated from the bark and needles of several *Taxus* plant species (Wall and Wani, 1995). Likewise, Artemisinin, a sesquiterpene isolated from the plant *Artemisia annua*, has been developed as a key pharmacological agent for the control of malaria (Tu, 2011). Patchouli, another sesquiterpene, is a popular aromatic found in colognes, perfumes and many other household cleaning products (Wu et al., 2006). Menthol is a monoterpene obtained from mint family plants and is a popular ingredient in many foods and consumer products (Bedoukian, 1983). Triterpenes such as squalene, obtained from various plant sources and the livers of deep sea sharks, have utility as a nutraceutical product, is used extensively in many types of cosmetics, has special utility as a lubricant for high performance machinery, and is a common adjuvant in many pharmaceutical formulations (Huang et al., 2009; Reddy and Couvreur, 2009; Bhilwade et al., 2010).

Terpenes are, however, generally made by plants and microbes in small amounts and components of complex mixtures that vary with growth and environmental conditions, making it difficult to reproducibly obtain large amounts of any one terpene constituent (Wu et al., 2006). Chemical synthesis of terpenes is often costly and inefficient (Nicolau et al., 1994). Chemical synthesis also suffers from generating enantiomeric mixtures, which adds other complications if one particular stereochemical form of a terpene is desired. Given such difficulties, there are many on-going efforts to create robust, reliable and efficient biological systems for the production of distinct classes of terpenes, and more so for the generation of stereochemically pure forms of terpenes (Martin et al., 2003; Wu et al., 2006;

Takahashi et al., 2007; Asadollahi et al., 2008; Kirby et al., 2008; Seki et al., 2008; Keasling, 2009; Asadollahi et al., 2010; Fischer et al., 2011). The current invention disclosure describes the generation of yeast lines that we claim have utility for the production of diverse classes of terpenes including monoterpenes, sesquiterpenes, diterpenes and triterpenes.

Diterpenes are a class of compounds within the much larger terpene family of molecules (FIG. 4). Terpenes, in general, are built upon a 5 carbon repeating unit giving rise to classes of compounds having 10 (monoterpenes), 15 (sesquiterpenes), 20 (diterpenes), and more carbons. The current disclosure pertains to diterpenes, which are known to have diverse biological and practical applications. In plants, specific diterpenes serve as hormones or growth regulators (i.e. gibberellic acid derivatives) (Yamaguchi, 2008) while others serve as accessory photo-pigments funneling energy from light capture to the light reactions of photosynthesis (Havaux et al., 2004). Other diterpenes provide protection against oxidative radicals (Grassmann, 2005). The antioxidant activity of diterpenes has also led to their use in human nutraceuticals and medical applications (Cardenas et al., 2011). Perhaps the most widely recognized diterpene is taxol, used very successfully and extensively for the treatment of a variety of cancers (Wall and Wani, 1995). Specific diterpenes have also found use in the control of dental caries providing antimicrobial activities (Porto et al., 2009). Other diterpenes have found utility in manufacturing purposes, such as in the production of tackifiers (U.S. Pat. No. 7,655,739), herein incorporated by reference.

Diterpenes are traditionally obtained from plant sources. However, they are often found in only small amounts and as components of complex mixtures that vary with growth and environmental conditions, making it difficult to obtain large amounts of any one diterpene constituent (Wu et al., 2006). Chemical synthesis of diterpenes is often costly and inefficient (Nicolaou et al., 1994). Chemical synthesis also suffers from generating enantiomeric mixtures, which adds other complications if one particular stereochemical form of a terpene is desired. Given such difficulties, there are many on-going efforts to create robust, reliable and efficient biological systems for the production of distinct diterpenes, and more so for the generation of stereochemically pure forms of diterpenes (DeJong et al., 2006; Kovacs et al., 2007; Roberts, 2007; Engels et al., 2008; Anterola et al., 2009). The current invention disclosure describes the generation of yeast lines that we claim have utility for the production of diverse and high-value diterpenes.

SUMMARY OF THE INVENTION

The presently disclosed subject matter relates to methods for producing modified yeast cell lines to produce "platforms" in yeast and the resulting modified yeast or platforms. The production platforms can be further modified to produce specific terpenes such as diterpenes. Advantageously, the method includes modifying yeast to express avian farnesyl diphosphate synthase and preferably mutant avian farnesyl diphosphate synthase. The modification advantageously is provided by an expression vector encoding mutant avian farnesyl diphosphate synthase. The expression vector can be inserted into wildtype yeast including but not limited to *Candida albicans* (*C. albicans*) and *Saccharomyces cerevisiae* (*S. cerevisiae*). These can be ergosterol (hereinafter "erg") erg- or erg+ and/or have sterol uptake enhancement (hereinafter "SUE") SUE+ or SUE-. Especially advantageous yeast platforms are both erg- and SUE-

The mutant avian farnesyl diphosphate synthase (mtFPS) converts dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP) to geranylgeranyldiphosphate (GGPP) and from GGPP to various desired diterpenes.

The present invention, in one form, relates to genetically modified yeast which expresses mtFPS. The yeast may be erg+ or erg- and/or SUE+ or SUE-.

The present invention, in another form thereof relates to a method for producing a genetically modified yeast comprising inserting an expression vector into a yeast cell wherein the expression vector expresses a gene for mtFPS.

The present invention, in another form thereof relates to a method for generating terpene produced yeast cell lines. The method includes combining yeast with a chemical mutagenesis agent to induce mutations in the yeast to generate chemically modified yeast. The chemically modified yeast are selected which grow in the presence of nystatin, squalestatin, and cholesterol followed by selecting for ergosterol dependent growth. The ergosterol dependent growth yeast are subjected to an erg9 knockout mutation to thereby produce ergosterol dependent growth/erg9 knockout mutation yeast cell lines. An expression vector is inserted into the ergosterol dependent growth/erg9 knockout mutation yeast cells wherein the expression vector expresses a gene for mtFPS.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 shows biosynthesis of terpenes from natural sources, often in mixtures, produced by wild type yeast.

FIG. 2 is schematic outline of two terpene biosynthetic pathways that operate in plants (the MVA and MEP pathways), their intracellular locations, and examples of the chemical compounds derived from each.

FIG. 3 illustrates mevalonate pathway in ergosterol biosynthesis in yeast (*S. cerevisiae*).

FIG. 4 is a schematic representing compounds of various terpenoid classes and prenyl diphosphates derived.

FIG. 5 is a schematic showing one strategy for developing a yeast line suitable for engineering diterpene chemicals in accordance with the present invention.

FIG. 6 is a graph showing a comparison of terpene chemical profiles in yeast over-expressing the abietadiene synthase (ABS) gene versus control yeast not harboring the ABS gene.

FIG. 7 is a graph showing a comparison of terpene chemical profiles from yeast co-expressing an alternative GGPP synthase (mFPS F112A) with abietadiene synthase versus only over-expressing the abietadiene synthase.

FIG. 8 comprises two graphs showing co-expression of the mFPS gene with different diterpene synthase genes enhances diterpene accumulation.

FIG. 9 illustrates a construct design for testing the importance of specific gene promoters for diterpene (kaurene) production in yeast.

FIG. 10 comprises four graphs showing kaurene and farnesol (FOH) accumulation in yeast engineered for expression of the kaurene synthase and mutant avian FPP synthase driven by different gene promoters.

FIG. 11 shows a comparison of vector configuration effects on abietadiene production in ZX 2-2 yeast line.

FIG. 12 is a chart showing kaurene, farnesol (FOH) and geranylgeraniol accumulation in yeast engineered for expression of kaurene synthase and mutant avian FPP synthase driven by different gene promoter combinations on a single gene expression vector.

FIG. 13 comprises two charts show culture medium and temperature influences on abietadiene accumulation.

FIG. 14 shows expression constructs designed for producing diterpene acids in yeast in accordance with the present invention.

FIG. 15 is a chart showing culture medium and temperature influence kaurenoic acid accumulation in accordance with the present invention.

DETAILED DESCRIPTION

The present method and modified yeast will now be described with reference to the figures and exemplary experiments, examples and methods. The figures, experiments and examples are merely to provide a more thorough understanding of the present method and modified yeast. However, other methods and generated yeast can be envisioned consistent with the scope and spirit of this disclosure.

FIG. 5 outlines one approach used to generate yeast lines that provides for robust biosynthesis of precursors that can be utilized for the production of many different classes of terpenes. The schematic diagram in FIG. 5 shows an overall approach used for generating a yeast cell line that have a dispensable sterol biosynthetic pathway (FIG. 5, upper panel A), which provide opportunities for diverting intermediaries (DMAPP, IPP and FPP) from the mevalonate (MVA) pathway for the biosynthesis of diterpene compounds (FIG. 5, lower panel B).

The strategy takes advantage of the native mevalonate (MVA) pathway that operates normally in yeast for the biosynthesis of ergosterol, the dominant sterol found in yeast. Ergosterol is the main product of the yeast mevalonate pathway, is an important membrane component, and is essential for yeast growth. If the ergosterol biosynthetic pathway is blocked or inhibited, yeast die. In fact, this is the basis for many pharmacological drugs to control fungal infections in man (Maertens, 2004) and agricultural chemicals to control fungal infection in plants (Casida, 2009). To further complicate matters, wild type yeast can take up exogenously supplied sterol from their environment only under anaerobic conditions.

In order to be able to efficiently channel terpene biosynthetic intermediates from the ergosterol biosynthetic pathway (FIG. 5, panel A), a SUE (sterol uptake enhancement) mutation supporting the aerobic uptake and utilization of exogenous sterol was first created (Bourot and Karst, 1995; Shianna et al., 2001). A SUE mutation is thus a yeast line that can meet all its sterol needs by an exogenous source of sterol, and therefore making the endogenous ergosterol biosynthetic pathway dispensable (Bourot and Karst). The Bourot and Karst SUE mutation was then complemented by the introduction of a knockout mutation in the ERGS gene (squalene synthase) (Zhang et al., 1993), resulting in a yeast line where the MVA pathway was still operational up to the biosynthesis of FPP and hence, intermediates in the pathway (DMAPP, IPP and FPP) could be diverted to the biosynthesis of other non-essential terpene components.

This technique diverts isoprenoid pathway intermediates to the biosynthesis of diterpenes, to provide high yielding conditions for the production of diterpene hydrocarbons and decorating the diterpene scaffolds to generate additional high-valued chemical entities.

Steps in the Development of High Level Diterpene Accumulation in Yeast

I. Co-Expression of a Mutant Prenyltransferase

Specific efforts and conditions were necessary to generate yeast lines expressing high-level diterpene accumulation.

The first was based on the observation that yeast engineered with a diterpene synthase, like abietadiene synthase, ABS (Vogel et al., 1996) tend to accumulate only marginal amounts of the desired diterpene product (FIG. 6). However, when expression of the ABS gene is coupled with the co-expression of a mutant avian farnesyl diphosphate synthase (mtFPS) that exhibits a preferred biosynthesis for geranylgeranyl diphosphate (GGPP) rather than FPP (Tarshis et al., 1996; Fernandez et al., 2000), those yeast lines demonstrated a dramatic accumulation of abietadiene at the expense of farnesol accumulation (FIG. 7). Use of the mtFPS was preferred to other native GGPP synthases because the avian enzyme is particular active as a homodimeric protein and because the enzyme protein is itself relatively small.

Referring to FIG. 6, GC chromatographs of extracts were prepared from yeast engineered for expression of the abietadiene synthase (ABS) gene (upper panel) versus control yeast (those engineered with an empty plasmid DNA, no ABS gene) (lower panel). The yeast lines were grown for approximately 5 days, aliquots of the culture were extracted into hexane, and the hexane extracts then profiled by GC-MS. Cedrene was added to cultures prior to extraction as an external standard to account for sample extraction efficiency (peak 1); farnesol (peak 3) was monitored as an estimation of how much carbon flux to FPP was occurring in the yeast cells; and abietadiene (peak 5) was monitored as a measure of how much isoprenoid intermediates (IPP, DMAPP and FPP) were being diverted to diterpene biosynthesis.

FIG. 7 provides data from GC chromatograms of yeast co-expressing the abietadiene synthase (ABS) and a mutant avian farnesyl diphosphate synthase (mtFPS) (upper panel) versus a yeast line only expressing the ABS gene (lower panel). The yeast lines were grown for approximately 5 days, aliquots of the culture were extracted into hexane, and the hexane extracts then profiled by GC-MS. Cedrene was added to cultures prior to extraction as an external standard to account for sample extraction efficiency (peak 1); farnesol (peak 2) was monitored as an estimation of how much carbon flux to FPP was occurring in the yeast cells, thus escaping channeling to diterpene biosynthesis; and abietadiene (peak 3) was monitored as a measure of how much isoprenoid intermediates (IPP, DMAPP and FPP) were being diverted to specialized diterpene biosynthesis.

FIG. 8 comprises graphs for assessing if the co-expression of the mtFPS gene with different diterpene synthases genes enhances diterpene accumulation. In the upper panel of FIG. 8, yeast engineered for expression of the ABS gene or ABS gene plus mtFPS gene were grown under standard conditions and aliquots of the cultures were monitored daily for growth (OD600 nm) and abietadiene accumulation (GC-MS determination). In the lower panel of FIG. 8, yeast were engineered for expression of a second diterpene synthase gene, kaurene synthase, plus and minus co-expression of the mtFPS gene. Cultures were monitored daily for growth (OD600 nm) and kaurene accumulation (GC-MS).

The benefit of co-expressing the mutant FPS gene with other diterpene synthases for the improved yield of diterpene hydrocarbons was examined with other diterpene synthase genes as well. In FIG. 8, co-expression of the mtFPS gene along with a codon optimized fungal kaurene synthase gene (Toyomasu et al., 2000) dramatically improved kaurene accumulation (lower panel) as observed for abietadiene biosynthesis (upper panel). Equally important to note, the enhanced diterpene accumulation due to the co-expression of the mtFPS did not impose any obvious penalty in cell biomass accumulation (OD600 nm). Cell culture growth

was, in fact, improved from 20 to 40% when the diterpene synthase genes were co-expressed with the mutant prenyltransferase.

II. Identification of Gene Expression Promoters and Vector Configurations to Enhance Diterpene Accumulation

The co-expression of the mutant FPS and diterpene synthases provides evidence that the expression level of each gene relative to one another (the stoichiometric relationship) might be an important for optimized diterpene production

FIG. 9 is a schematic showing a construct design for testing the importance of specific gene promoters for diterpene (kaurene) production in yeast. A variety of promoter elements were inserted independently upfront of the mtFPS gene and the fungal kaurene synthase gene followed by the yeast being transformed with all possible combinations of each construct. The different transgenic yeast lines would then evaluate for kaurene production levels.

A variety of gene promoter combinations were evaluated for determining the regulation level of target enzymes in the yeast cells as shown in the strategy outline in FIG. 9.

As shown in FIG. 9, the promoter elements included the actin (*act*) promoter (Mateus and Avery, 2000), a copper inducible (*cup*) promoter (Tohoyama et al., 2001), glyceraldehyde phosphate dehydrogenase (*gpd*) promoter (Bitter and Egan, 1984), transcription elongation factor (*tef*) promoter (Mumberg et al., 1995), and the alcohol dehydrogenase (*adh*) promoter, which we previous described using for heterologous expression in yeast (Takahashi et al., 2007). Yeast strain ZX 2-2 was co-transformed with the various two plasmid construct combinations, then individual transformant lines were monitored for kaurene and farnesol accumulation (FIG. 10). While we were obviously screened these lines for the promoter combination giving the highest level of diterpene production, an equal important parameter was the farnesol levels. If a yeast line was efficiently diverting the earlier isoprenoid precursors to diterpene, their farnesol levels would be expected to be equally low. By these criteria, having the GPD promoter direct expression of both the mutant prenyltransferase and the kaurene synthase genes yielded the highest level of kaurene with the greatest efficiency.

The data in the graphs of FIG. 10 were from yeast transformed with the various plasmids noted in FIG. 9, selected for prototrophic growth without leucine or histidine added to the culture media, then grown for 10 days before extracting and chemically profiling aliquots of the cultures by GC-MS. For the line harboring the CUP promoter construct, the cultures were grown for 2 days, then 1 mM copper sulfate was added to the growth media.

The results of FIG. 10 demonstrated that the absolute level of gene expression and stoichiometry of the encoded enzymes influenced overall diterpene production. Next the prenyltransferase and diterpene synthase genes were assembled in separate plasmid vectors or into a single vector. In this way, we were evaluating whether variation in diterpene accumulation could be associated with possible variation in gene copy number as reflected by possible variation in plasmid copy number, or whether a one-to-one stoichiometry of prenyltransferase and diterpene synthase genes on a single plasmid vector were preferable. In the first examination of these possibilities, the constructs relied on the GPD promoter to drive expression of the prenyltransferase and diterpene synthase genes. The constructs were then introduced into yeast and multiple, independent transformants selected for monitoring diterpene (abietadiene) production and farnesol accumulation (FIG. 11). Farnesol accumulation was monitored as a measure of how much carbon was not

efficiently being converted to diterpene. Surprisingly, those transgenic lines with the multiple plasmid constructs exhibited relatively minor variation in the level of diterpene and farnesol accumulated, while the lines transformed with the single vector harboring both the prenyltransferase and diterpene synthase genes showed more than 50% variation in the absolute levels of farnesol and abietadiene. Nonetheless, independent transgenic lines containing the targeted genes on a single plasmid vector also demonstrated greater than 30% more abietadiene and farnesol than when the transgenes were introduced on separate plasmid vectors.

FIG. 11 provides data from test showing how the molecular configuration of the mutant prenyltransferase (mtFPS, geranylgeranyl diphosphate synthase) relative to the diterpene synthase (ABS, abietadiene synthase) might influence diterpene production. The indicated plasmid vectors were transformed into yeast ZX 2-2 and 4 independent transgenic lines grown for 10 days prior to extracting and chemically profiling the extracts by GC-MS for abietadiene and farnesol (FOH) accumulation.

The most optimal vector design suggested by the experimental work up to this point suggest that having both the prenyltransferase and diterpene synthase genes on one plasmid vector and having expression of both genes driven by the GPD promoter was the preferred structural organization. This was confirmed in another experiment where the GPD promoter elements within the single plasmid construct were substituted with the ACT, ADH and TEF promoter elements and the transgenic lines examined for farnesol and kaurene accumulation (FIG. 12). Once again, the combination of the dual combination of the GPD promoters proved superior to any other promoter combination with respect to kaurene yield and efficiency, as noted by the limited amount of farnesol accumulating.

The graph of FIG. 12 provides data from evaluating the efficiency of the ACT, ADH, TEF or GPD promoters to direct expression of both the mutant prenyltransferase and kaurene synthase genes on a single plasmid vector for diterpene production. Yeast line ZX 2-2 was transformed with the indicated plasmid vector and a resulting transformant line chemically profiled by GC-MS after 10 days of growth.

III. Optimization of Culture Conditions to Enhance Diterpene Accumulation

During the evaluation of genes and genetic elements for enhancing diterpene accumulation, variation in diterpene yields with the culture conditions were observed upon examination of these parameters more systematically, it was discovered that for each diterpene target, specific culture conditions could dramatically influence overall diterpene accumulation. In FIG. 13, the accumulation of abietadiene, farnesol and geranylgeraniol by the same yeast line over-expressing the mutant prenyltransferase and abietadiene synthase genes grown under 4 conditions (2 temperatures and 2 media) was examined. Not unexpectedly, when grown in nutrient rich media (YPDE), the yeast grew approximately 2-fold greater than when cultured in selection media (SCE) for 10 days. However, growth of the cultures at 23° C. versus 30° C. had relatively little influence over this 10-day period in terms of overall biomass accumulation. However, a dramatic effect on abietadiene accumulation was noted when the cultures were grown at 23° C. in selection media. Abietadiene accumulation was 2-fold greater under these conditions than when grown at the higher temperature or in the nutrient rich media. The latter observation might be explained by the loss of the recombinant expression plasmid from the yeast grown in the absence of selection pressure

provided by the selection media. In contrast, kaurene accumulation by yeast co-expressing the fungal kaurene synthase and mutant prenyltransferase also under the control of GPD promoters was highest in yeast grown in nutrient rich media rather than selection media, even if the cultures were grown for 10 or more days. Cooler culture temperatures appear to improve diterpene accumulation regardless of the diterpene synthase gene used.

FIG. 13 provides data correlating culture media and temperature influence diterpene accumulation. Yeast strain ZX178-08 co-expressing ABS and mtFPS under the direction of the GPD promoter was grown in nutrient rich media (YPDE) or selection media (SCE) at 23° C. or 30° C. for 10 days. Culture growth was then measure at OD600 nm and terpene accumulation was determined by GC-MS.

IV. Decorating Diterpene Hydrocarbon Scaffolds

Having achieved the production of diterpene hydrocarbon production in yeast, more highly modified forms of diterpenes and especially those molecules that might have industrial, agricultural or medicinal applications were sought. For this purpose, we have utilized a 3 plasmid construct design (FIG. 14). Plasmids 1 and 2 are those described above and whose expression in yeast yields robust levels of diterpenes such as kaurene. The third plasmid construct was similarly designed to constructs 1 and 2, but contained a gene encoding for kaurene oxidase, a fungal P450 enzyme (Tudzynski et al., 2001) requiring reducing equivalents from a cytochrome P450 reductase (CPR) (Takahashi et al., 2007) for activity.

These three vector constructs were transformed into yeast line 2-2 and a confirmed transformant evaluated for diterpene production at 23° C. and 30° C. in nutrient rich media and selection media as described before (FIG. 15). The yeast were grown for 10 days before the accumulation of kaurene and its specific oxidation products kaurenal and kaurenoic acid were measured by GC-MS. Consistent with the earlier observations for kaurene production only, maximal production of approximately 200 mg/l of kaurenoic acid was determined for the culture grown in nutrient rich media at the reduced temperature. This diterpene productivity was about 2-fold greater than the next best conditions, which was the same lower temperature with selection growth media.

FIG. 14 is a schematic showing a construct design for producing diterpene acids in yeast in accordance with the present invention. The mutant prenyltransferase (mtFPS) and kaurene synthase (FKS) constructs were described above and the new construct consisting of a gene encoding for a fungal P450 enzyme catalyzing the oxidation of kaurene to it acidic form (kaurene oxidase, GFKO), plus a cytochrome P450 reductase (CPR) that provides reducing equivalents to the kaurene oxidase. Expression of these genes, like the prenyltransferase and kaurene synthase genes, is controlled by the GPD promoter.

FIG. 15 shows kaurenoic acid production in yeast is media and temperature sensitive. Yeast strain 2-2 was transformed with the 3 plasmid constructs shown in FIG. 14 and a single confirmed transformant grown under the conditions noted. The diterpene profile was determined after 10 days of growth by GC-MS analysis.

The following experiments, methods and procedures provide additional background with regard to the method for producing diterpene platforms in yeast and the resulting yeast produced. In addition, method for producing various knockout mutations in yeast are described in co-pending U.S. Patent Application Serial No.: 14/092,496, herein incorporated by reference.

The following disclosure provides and demonstrates utility of the yeast lines produced in accordance with the present disclosure for diterpene production via a bioreactor scale-up procedure.

Materials and Methods

Chemical and Media Preparations

All chemical reagents were obtained from Sigma-Aldrich (St. Louis, Mo.), BD Bioscience (Franklin Lakes, N.J.), or Fisher Scientific (Chicago, Ill.), while reagents for molecular manipulations were from Stratagene (San Diego, Calif.), Takara (Shiga, Japan), Invitrogen (San Diego, Calif.), and New England Biolab (Ipswich, Mass.).

Bacteria and yeast were grown using standard culture practices. YPD media for growing yeast without selection consisted of 1% Bactoyeast extract, 2% Bacto-peptone, and 2% glucose (or 0.5% glucose for select experiments). YPDE media was YPD media supplemented with ergosterol (40 mg/L) for ergosterol dependent lines. Minimal media, SCE (pH 5.3), contained 0.67% Bacto-yeast nitrogen base (without amino acids), 2% dextrose, 0.6% succinic acid, 0.14% Sigma yeast dropout solution (-his,-leu,-ura,-trp), uracil (300 mg/L), L-tryptophan (150 mg/L), L-histidine (250 mg/L), L-methionine (200 mg/L), L-leucine (1g/L) and 40 mg/L ergosterol. Cholesterol and ergosterol stocks were 10 mg/mL in 50% Triton X-100, 50% ethanol and kept at -20° C. Selection media was prepared similarly except without supplementing the media with the indicated reagent based on the yeast auxotrophic makers. All solid media plates were prepared with 2% Bacto-Agar.

Yeast Strains

The ZX yeast lines used in these studies were disclosed previously. Essentially, these strains were selected for their ability to utilize exogenous sterol sources under aerobic conditions and were engineered with a knockout mutation in their squalene synthase (ERGS) gene such that the basic mevalonate biosynthetic pathway is operative up to FPP biosynthesis. In some cases, similarly constructed yeast strain CAL17 was utilized (Takahashi et al., 2007).

Yeast Transformation and Culture Performance

Yeast strains were transformed with the respective vector constructs using the FROZEN-EZ Yeast Transformation II Kit (Zymo Research, Orange, Calif.) according to the manufacturer's recommendations. About 1 pg of plasmid DNA was used per transformation, followed by selection on agar plates of SCE medium lacking specified amino acids for the auxotrophic markers (selection media) or YPDE (rich media) at 30° C. Variable numbers of independent colonies were subsequently picked and used to start 3 ml cultures in minimal media to characterize their terpene production capacities. Aliquots of these cultures were analyzed for terpene production after 3-6 days of incubation at 30° C. with shaking by GC-MS. Cultures exhibiting the highest terpene production levels were chosen for further studies and archived as glycerol stocks at -80° C. Selected lines were characterized for cell growth and terpene production using 30 mL shake flask cultures. Starter cultures grown to saturation in minimal media were inoculated into 30 ml SCE or YPDE media and 1 mL aliquots withdrawn at indicated intervals for up to 15 days. Cell growth was monitored as the change in optical density at 600 nm, using appropriate dilutions for cultures at later stages of growth. Terpene production was determined by GC-MS similar to the initial screening method.

GC-MS Detection and Quantification of Terpenes

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

Expression Vector Construction

The yeast GPD promoter (Pgpd) was amplified from the PYM-N14 plasmid described by Janke et al. (Janke et al., 2004) using the primers GPD-BamHIF and GPD-NotIR primers and inserted into the pESC-His vector digested with BamH1 and NotI to replace the original GAL1/10 promoters. The resulting plasmid was named pESC-His-gpd. The other promoter elements were obtained similarly.

It will be understood that various details of the presently disclosed subject matter can be changed without departing from the scope of the subject matter disclosed herein. Furthermore, the foregoing description is for the purpose of illustration only, and not for the purpose of limitation.

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SEQUENCE LISTING

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          35          40          45

Asp Thr Ala Trp Val Ala Met Ile Pro Lys Thr Arg Asp Asn Val Lys
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Gln Trp Leu Phe Pro Glu Cys Phe His Tyr Leu Leu Lys Thr Gln Ala
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115 120 125

Glu His Gly Val Thr Ser Leu Lys Arg Gln Leu Ala Val Trp Asn Asp
130 135 140

Val Glu Asp Thr Asn His Ile Gly Val Glu Phe Ile Ile Pro Ala Leu
145 150 155 160

Leu Ser Met Leu Glu Lys Glu Leu Asp Val Pro Ser Phe Glu Phe Pro
165 170 175

Cys Arg Ser Ile Leu Glu Arg Met His Gly Glu Lys Leu Gly His Phe
180 185 190

Asp Leu Glu Gln Val Tyr Gly Lys Pro Ser Ser Leu Leu His Ser Leu
195 200 205

Glu Ala Phe Leu Gly Lys Leu Asp Phe Asp Arg Leu Ser His His Leu
210 215 220

Tyr His Gly Ser Met Met Ala Ser Pro Ser Ser Thr Ala Ala Tyr Leu
225 230 235 240

Ile Gly Ala Thr Lys Trp Asp Asp Glu Ala Glu Asp Tyr Leu Arg His
245 250 255

Val Met Arg Asn Gly Ala Gly His Gly Asn Gly Gly Ile Ser Gly Thr
260 265 270

Phe Pro Thr Thr His Phe Glu Cys Ser Trp Ile Ile Ala Thr Leu Leu
275 280 285

Lys Val Gly Phe Thr Leu Lys Gln Ile Asp Gly Asp Gly Leu Arg Gly
290 295 300

Leu Ser Thr Ile Leu Leu Glu Ala Leu Arg Asp Glu Asn Gly Val Ile
305 310 315 320

Gly Phe Ala Pro Arg Thr Ala Asp Val Asp Asp Thr Ala Lys Ala Leu
325 330 335

Leu Ala Leu Ser Leu Val Asn Gln Pro Val Ser Pro Asp Ile Met Ile
340 345 350

Lys Val Phe Glu Gly Lys Asp His Phe Thr Thr Phe Gly Ser Glu Arg
355 360 365

Asp Pro Ser Leu Thr Ser Asn Leu His Val Leu Leu Ser Leu Leu Lys
370 375 380

Gln Ser Asn Leu Ser Gln Tyr His Pro Gln Ile Leu Lys Thr Thr Leu
385 390 395 400

Phe Thr Cys Arg Trp Trp Trp Gly Ser Asp His Cys Val Lys Asp Lys
405 410 415

Trp Asn Leu Ser His Leu Tyr Pro Thr Met Leu Leu Val Glu Ala Phe
420 425 430

Thr Glu Val Leu His Leu Ile Asp Gly Gly Glu Leu Ser Ser Leu Phe
435 440 445

Asp Glu Ser Phe Lys Cys Lys Ile Gly Leu Ser Ile Phe Gln Ala Val
450 455 460

Leu Arg Ile Ile Leu Thr Gln Asp Asn Asp Gly Ser Trp Arg Gly Tyr
465 470 475 480

Arg Glu Gln Thr Cys Tyr Ala Ile Leu Ala Leu Val Gln Ala Arg His
485 490 495

Val Cys Phe Phe Thr His Met Val Asp Arg Leu Gln Ser Cys Val Asp

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Lys Leu Phe Cys Asp Val Thr Asp Leu Tyr Asp Gln Leu Tyr Val Ile
 930 935 940

Lys Asp Leu Ser Ser Ser Met Lys
 945 950

<210> SEQ ID NO 9
 <211> LENGTH: 2868
 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: engineered sequence

<400> SEQUENCE: 9

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gatttcggtt ctgctgctaa atccttggtg gatagagctt ttaaatctca tcattcttac 120
tacggtttgt gttctacttc ttgtcaagtt tacgatactg cttgggttgc tatgattcca 180
aagactagag ataacggttaa gcaatgggtg ttcccagaat gttccatta tttggtgaaa 240
actcaagctg ctgatgggtc ttgggggttc ttgccaaacta ctcaaactgc tggatatttg 300
gatactgctt ctgctgtttt ggctttgttg tgcctgctc aagaaccatt gcaaatattg 360
gatgtttctc cagatgaaaat gggtttgaga attgaacatg gtgttacttc tttgaaaaga 420
caattggctg tttggaatga tgttgaagat actaatcata ttggtgttga gttcatcatt 480
ccagctttgt tgtctatggt ggaaaaagaa ttggatgttc catcttttga atttccatgt 540
agatctatct tggaaaagaat gcatgggtgaa aaattgggtc attttgattt ggaacaagtt 600
tatggtaaac catcttcttt gttgcattct ttggaagcat tccttggtaa attggatttt 660
gatagattgt ctcatcattt gtatcatggt tctatgatgg cttctccatc ttctactgct 720
gcttatttga ttgggtctac taaatgggat gatgaagctg aagattattt gagacatggt 780
atgagaaatg gtgctgggtc tggtaatggt ggtatttctg gtacttttcc aactactcat 840
ttcgaatggt cttggatcat cgctactttg ttgaaggttg gttttacttt gaagcaaatt 900
gatggagatg gtttgagagg tttgtctact attttggttg aagctcttag agatgaaaat 960
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gctttgtctt tggttaacca accagtttct ccagatatca tgatcaaggt tttcgagggt 1080
aaagatcatt tcaactactt cgggtctgaa agagatccat ctttgacttc taatttgcatt 1140
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tgtttcttta ctcatatggt tgatagattg caatcttgtg ttgatagagg tttttcttgg 1560
ttgaaatctt gttcttttca ttctcaagat ttgacttggg cttctaaaac tgettatgaa 1620
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tacatgagat tggttagaaa gactgctttg ttttctccat tggatgaatg gggtttgatg 1800
gcttctatta ttgaatcttc attttctgtt ccattgttgc aagctcaaag agttgaaatc 1860
    
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taccaagag ataacatcaa ggttgatgag gataagtatt tgtctatcat tccattcact	1920
tgggttggtt gtaacaacag atctagaact ttcgcttcta acagatgggt gtaacgatatg	1980
atgtatttgt ctttgttggg ttaccaaact gatgaatata tggaaactgt tgcgtgtcca	2040
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tctgatgctt tttctctccc agaacaatct tattttcaat gggtaattc tactgggtgt	2400
tctcatgttg cttgtgctta ttcttttctt ttttctaatt gtttgatgct tgctaatttg	2460
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gctgaaagaa acgttaattc tatccatttc ccagagttca ctttgtgtaa cggtagcttc	2640
caaaatttgg atgaaagaaa ggaaagattg ttgaagatcg ctacttacga acaaggttat	2700
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ggttctaagg atatgagaaa attgaagatc gttaaattgt tttgtgatgt tactgatttg	2820
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<210> SEQ ID NO 10

<211> LENGTH: 2115

<212> TYPE: DNA

<213> ORGANISM: *Artemisia annua*

<400> SEQUENCE: 10

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ggcaaggat ccttcgacac atcaaacaca tccgatacga atattccggt agccgtgttt	120
atggagaate gtgagctttt gatgatttta actacttcgg ttgcgggtct gatcggatgc	180
gttgtggtgc ttgtgtggag acggtcgtcg tcggcggcga agaaagcggc ggagtcgccg	240
gtgattgttg taccgaagaa agtgacggag gatgaggttg atgatggacg gaagaaagtt	300
actgtgtttt ttggaactca gactggtact gctgaaggtt ttgctaaggc gcttgttgaa	360
gaggctaaag cgcgatatga aaaggcggtg tttaaagtga ttgatttga tgattatgcc	420
gctgaagatg atgagatgga ggagaagta aagaaagaat ctcttgcctt tttctttta	480
gctacgtatg gagatggtga gccgacagat aatgctgcta gattctataa atggtttacc	540
gagggtgaag agaaaggtga atggcttgac aagcttcaat acgcagtgtt tggacttgg	600
aacagacagt atgagcattt caacaagatt gctaaggtgg tcgatgaaaa acttgtggaa	660
cagggtgcaa agcgccttgt tcctgttggc atgggagacg atgatcaatg tatcgaagac	720
gacttcactg catggaagaa gttggtgtgg cctgagttgg atcaattact tctgtgatgag	780
gatgatacat ctgttgccac tccatacaca gctgctgttg cagaataccg tgttgtgttc	840
catgataaac cagagacata tgatcaggat caactgacaa atggccatgc tgttcatgat	900
gctcaacatc catgcagatc caatgtcgtc gtcaaaaagg agctccattc ccctctatct	960
gaccggtcct gactcattt ggaatttcat atctctaata ctggattatc gtatgaaact	1020
ggggaccatg ttggagtcta tgttgagaat ctaagtgaag ttgtggacga agctgaaaaa	1080
ttaataggtt taccgccga cacttatttc tcagtacaca ctgataacga agacgggaca	1140

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ccacttggtg gagcatcttt gccacctcct ttcctccat gcactttaag aaaagcattg 1200
gcttcctatg ctgatgtttt gagctctcct aaaaagtcag ctttgcttgc tttagctgct 1260
catgccactg atttactga agctgataga ctgaaatttc ttgcgtctcc tgctggaag 1320
gatgaatatg ctcagtggat agttgcaagc cacagaagtc tccttgaggt catggaggcc 1380
ttcccatcag ctaagcctcc gcttggtgtt ttttttgcac ctggtgcccc acgtttgcag 1440
ccgagatact attccatttc ttcttcccca aagtttgcgc caaataggat tcatgtaact 1500
tgtgcattag tgatagagca aacaccgtca ggccgcgttc acaagggagt ctgttcaaca 1560
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gttagaacat ccaatttcag acttccttct gatcctaagg tcccagttat catgattggc 1680
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ttcatatatg aagacgagct taataathtt gtggagacgg gggctctttc cgagcttgtt 1860
acggccttct ctctggaagg tgccactaag gagtacgtgc aacacaagat gactcagaag 1920
gcttcggata tctggaattt tctctctgag ggagcatatt tgtatgtttg cggatgatgcc 1980
aaaggcatgg ccaaagatgt acatcggact ctgcacacaa ttgtgcaaga acagggatct 2040
ctagactcct caaagcgga gctctacgtg aagaatctac aaatggcagg aagatatctc 2100
cgtgatgtat ggtaa 2115

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<210> SEQ ID NO 11
<211> LENGTH: 704
<212> TYPE: PRT
<213> ORGANISM: Artemisia annua

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<400> SEQUENCE: 11

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Met Gln Ser Thr Thr Ser Val Lys Leu Ser Pro Phe Asp Leu Met Thr
1           5           10          15
Ala Leu Leu Asn Gly Lys Val Ser Phe Asp Thr Ser Asn Thr Ser Asp
                20          25          30
Thr Asn Ile Pro Leu Ala Val Phe Met Glu Asn Arg Glu Leu Leu Met
            35          40          45
Ile Leu Thr Thr Ser Val Ala Val Leu Ile Gly Cys Val Val Val Leu
        50          55          60
Val Trp Arg Arg Ser Ser Ser Ala Ala Lys Lys Ala Ala Glu Ser Pro
        65          70          75          80
Val Ile Val Val Pro Lys Lys Val Thr Glu Asp Glu Val Asp Asp Gly
            85          90          95
Arg Lys Lys Val Thr Val Phe Phe Gly Thr Gln Thr Gly Thr Ala Glu
        100         105         110
Gly Phe Ala Lys Ala Leu Val Glu Glu Ala Lys Ala Arg Tyr Glu Lys
        115         120         125
Ala Val Phe Lys Val Ile Asp Leu Asp Asp Tyr Ala Ala Glu Asp Asp
        130         135         140
Glu Tyr Glu Glu Lys Leu Lys Lys Glu Ser Leu Ala Phe Phe Phe Leu
        145         150         155         160
Ala Thr Tyr Gly Asp Gly Glu Pro Thr Asp Asn Ala Ala Arg Phe Tyr
            165         170         175
Lys Trp Phe Thr Glu Gly Glu Glu Lys Gly Glu Trp Leu Asp Lys Leu
        180         185         190

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Gln Tyr Ala Val Phe Gly Leu Gly Asn Arg Gln Tyr Glu His Phe Asn
 195 200 205
 Lys Ile Ala Lys Val Val Asp Glu Lys Leu Val Glu Gln Gly Ala Lys
 210 215 220
 Arg Leu Val Pro Val Gly Met Gly Asp Asp Asp Gln Cys Ile Glu Asp
 225 230 235 240
 Asp Phe Thr Ala Trp Lys Glu Leu Val Trp Pro Glu Leu Asp Gln Leu
 245 250 255
 Leu Arg Asp Glu Asp Asp Thr Ser Val Ala Thr Pro Tyr Thr Ala Ala
 260 265 270
 Val Ala Glu Tyr Arg Val Val Phe His Asp Lys Pro Glu Thr Tyr Asp
 275 280 285
 Gln Asp Gln Leu Thr Asn Gly His Ala Val His Asp Ala Gln His Pro
 290 295 300
 Cys Arg Ser Asn Val Ala Val Lys Lys Glu Leu His Ser Pro Leu Ser
 305 310 315 320
 Asp Arg Ser Cys Thr His Leu Glu Phe Asp Ile Ser Asn Thr Gly Leu
 325 330 335
 Ser Tyr Glu Thr Gly Asp His Val Gly Val Tyr Val Glu Asn Leu Ser
 340 345 350
 Glu Val Val Asp Glu Ala Glu Lys Leu Ile Gly Leu Pro Pro His Thr
 355 360 365
 Tyr Phe Ser Val His Thr Asp Asn Glu Asp Gly Thr Pro Leu Gly Gly
 370 375 380
 Ala Ser Leu Pro Pro Pro Phe Pro Pro Cys Thr Leu Arg Lys Ala Leu
 385 390 395 400
 Ala Ser Tyr Ala Asp Val Leu Ser Ser Pro Lys Lys Ser Ala Leu Leu
 405 410 415
 Ala Leu Ala Ala His Ala Thr Asp Ser Thr Glu Ala Asp Arg Leu Lys
 420 425 430
 Phe Leu Ala Ser Pro Ala Gly Lys Asp Glu Tyr Ala Gln Trp Ile Val
 435 440 445
 Ala Ser His Arg Ser Leu Leu Glu Val Met Glu Ala Phe Pro Ser Ala
 450 455 460
 Lys Pro Pro Leu Gly Val Phe Phe Ala Ser Val Ala Pro Arg Leu Gln
 465 470 475 480
 Pro Arg Tyr Tyr Ser Ile Ser Ser Ser Pro Lys Phe Ala Pro Asn Arg
 485 490 495
 Ile His Val Thr Cys Ala Leu Val Tyr Glu Gln Thr Pro Ser Gly Arg
 500 505 510
 Val His Lys Gly Val Cys Ser Thr Trp Met Lys Asn Ala Val Pro Met
 515 520 525
 Thr Glu Ser Gln Asp Cys Ser Trp Ala Pro Ile Tyr Val Arg Thr Ser
 530 535 540
 Asn Phe Arg Leu Pro Ser Asp Pro Lys Val Pro Val Ile Met Ile Gly
 545 550 555 560
 Pro Gly Thr Gly Leu Ala Pro Phe Arg Gly Phe Leu Gln Glu Arg Leu
 565 570 575
 Ala Gln Lys Glu Ala Gly Thr Glu Leu Gly Thr Ala Ile Leu Phe Phe
 580 585 590
 Gly Cys Arg Asn Arg Lys Val Asp Phe Ile Tyr Glu Asp Glu Leu Asn
 595 600 605
 Asn Phe Val Glu Thr Gly Ala Leu Ser Glu Leu Val Thr Ala Phe Ser

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610	615	620
Arg Glu Gly Ala Thr Lys Glu Tyr Val Gln His Lys Met Thr Gln Lys		
625	630	635 640
Ala Ser Asp Ile Trp Asn Phe Leu Ser Glu Gly Ala Tyr Leu Tyr Val		
	645	650 655
Cys Gly Asp Ala Lys Gly Met Ala Lys Asp Val His Arg Thr Leu His		
	660	665 670
Thr Ile Val Gln Glu Gln Gly Ser Leu Asp Ser Ser Lys Ala Glu Leu		
	675	680 685
Tyr Val Lys Asn Leu Gln Met Ala Gly Arg Tyr Leu Arg Asp Val Trp		
	690	695 700

<210> SEQ ID NO 12
 <211> LENGTH: 1530
 <212> TYPE: DNA
 <213> ORGANISM: Arabidopsis thaliana

<400> SEQUENCE: 12

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atcttcttct tcaagaaact tctctccttc tccagaaaga acatgtctga agtctccact     120
ctcccctctg ttccagtggt accaggggtt cctgttattg ggaacttgct gcaactaaaa     180
gagaagaaac ctcacaagac ttctactaga tggtcagaga tttatgggtc tatttactct     240
ataaagatgg gttcttcttc tcttattgtc ctcaattcta ctgagactgc caaagaggcc     300
atggtgacgc ggttttcgct tatctcaacg aggaagttgt caaatgcggt gacagtcctt     360
acttgtgaca aatctatggt tgctactagt gattatgatg atttcacaa gttggtgaaa     420
cggtgtctct tgaacggctc tttgggtgct aatgcacaga aacgaaaaag acattacaga     480
gatgcactca ttgaaaaatgt gtcttccaag ttgcatgccc atgctaggga ccatccacaa     540
gaaacctgta acttcagagc tatatttgag catgagcttt tcggtgtagc attgaagcaa     600
gcttttggga aagatgtgga atccatttat gttaaagaac tcggtgtgac tttgtcgaaa     660
gacgagatct tcaaggtttt agtacatgac atgatggaag gtgcaattga tgttgattgg     720
agagacttct tcccatactt gaaatggatt ccaaataaaa gttttgaagc aagaatccag     780
caaaagcata aacgtagact cgcagtgatg aatgctctga ttcaagatcg actgaagcag     840
aatggttcag aatcggatga tgattgctat ctcaacttct tgatgtcgga agcgaaaaaca     900
ctaaccaagg agcaaatgc tatcttggtt tgggagacga ttatcgagac agctgacact     960
actttgggta caactgaatg ggccatctat gagctcgcta agcatccaag tgtccaagat    1020
cgtctgtgca aagaaatcca aaatgtctgc ggaggagaaa agttcaaaga agagcaattg    1080
tctcaagttc cttatctcaa tggagtcttt catgaaacgc ttaggaaata cagtctctgct    1140
cctctagttc ccattcgcta cgtccacgag gatacgcaaa tcggaggeta tcatgtccct    1200
gcaggaagtg agattgcaat aacatataat ggatgcaaca tggataagaa gcgttgggag    1260
agaccagagg actggtggcc ggagcgggtt cttgatgatg gcaaatatga aacgtcagat    1320
cttcacaaga caatggcgtt tggagcggga aagaggggtt gtgctggtgc tcttcaagca    1380
tctctcatgg caggcattgc tattggaaga ttagtgcaag aattcgagtg gaagcttaga    1440
gatggcgaag aagagaatgt ggatacatat ggcttgacct ctcagaagct ttatcctctt    1500
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<210> SEQ ID NO 13

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<211> LENGTH: 509
<212> TYPE: PRT
<213> ORGANISM: Arabidopsis thaliana

<400> SEQUENCE: 13

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 1                               5 10 15
Ser Phe Ile Phe Ile Phe Phe Phe Lys Lys Leu Leu Ser Phe Ser Arg
 20 25 30
Lys Asn Met Ser Glu Val Ser Thr Leu Pro Ser Val Pro Val Val Pro
 35 40 45
Gly Phe Pro Val Ile Gly Asn Leu Leu Gln Leu Lys Glu Lys Lys Pro
 50 55 60
His Lys Thr Phe Thr Arg Trp Ser Glu Ile Tyr Gly Pro Ile Tyr Ser
 65 70 75 80
Ile Lys Met Gly Ser Ser Ser Leu Ile Val Leu Asn Ser Thr Glu Thr
 85 90 95
Ala Lys Glu Ala Met Val Thr Arg Phe Ser Ser Ile Ser Thr Arg Lys
 100 105 110
Leu Ser Asn Ala Leu Thr Val Leu Thr Cys Asp Lys Ser Met Val Ala
 115 120 125
Thr Ser Asp Tyr Asp Asp Phe His Lys Leu Val Lys Arg Cys Leu Leu
 130 135 140
Asn Gly Leu Leu Gly Ala Asn Ala Gln Lys Arg Lys Arg His Tyr Arg
 145 150 155 160
Asp Ala Leu Ile Glu Asn Val Ser Ser Lys Leu His Ala His Ala Arg
 165 170 175
Asp His Pro Gln Glu Pro Val Asn Phe Arg Ala Ile Phe Glu His Glu
 180 185 190
Leu Phe Gly Val Ala Leu Lys Gln Ala Phe Gly Lys Asp Val Glu Ser
 195 200 205
Ile Tyr Val Lys Glu Leu Gly Val Thr Leu Ser Lys Asp Glu Ile Phe
 210 215 220
Lys Val Leu Val His Asp Met Met Glu Gly Ala Ile Asp Val Asp Trp
 225 230 235 240
Arg Asp Phe Phe Pro Tyr Leu Lys Trp Ile Pro Asn Lys Ser Phe Glu
 245 250 255
Ala Arg Ile Gln Gln Lys His Lys Arg Arg Leu Ala Val Met Asn Ala
 260 265 270
Leu Ile Gln Asp Arg Leu Lys Gln Asn Gly Ser Glu Ser Asp Asp Asp
 275 280 285
Cys Tyr Leu Asn Phe Leu Met Ser Glu Ala Lys Thr Leu Thr Lys Glu
 290 295 300
Gln Ile Ala Ile Leu Val Trp Glu Thr Ile Ile Glu Thr Ala Asp Thr
 305 310 315 320
Thr Leu Val Thr Thr Glu Trp Ala Ile Tyr Glu Leu Ala Lys His Pro
 325 330 335
Ser Val Gln Asp Arg Leu Cys Lys Glu Ile Gln Asn Val Cys Gly Gly
 340 345 350
Glu Lys Phe Lys Glu Glu Gln Leu Ser Gln Val Pro Tyr Leu Asn Gly
 355 360 365
Val Phe His Glu Thr Leu Arg Lys Tyr Ser Pro Ala Pro Leu Val Pro
 370 375 380
Ile Arg Tyr Ala His Glu Asp Thr Gln Ile Gly Gly Tyr His Val Pro

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385		390		395		400
Ala Gly Ser Glu Ile	Ala Ile Asn Ile Tyr Gly Cys Asn Met Asp Lys					
	405			410		415
Lys Arg Trp Glu Arg Pro Glu Asp Trp Trp Pro Glu Arg Phe Leu Asp						
	420			425		430
Asp Gly Lys Tyr Glu Thr Ser Asp Leu His Lys Thr Met Ala Phe Gly						
	435			440		445
Ala Gly Lys Arg Val Cys Ala Gly Ala Leu Gln Ala Ser Leu Met Ala						
	450			455		460
Gly Ile Ala Ile Gly Arg Leu Val Gln Glu Phe Glu Trp Lys Leu Arg						
	465			470		475
Asp Gly Glu Glu Glu Asn Val Asp Thr Tyr Gly Leu Thr Ser Gln Lys						
	485			490		495
Leu Tyr Pro Leu Met Ala Ile Ile Asn Pro Arg Arg Ser						
	500			505		

<210> SEQ ID NO 14
 <211> LENGTH: 1578
 <212> TYPE: DNA
 <213> ORGANISM: Gibberella fujikuroi

<400> SEQUENCE: 14

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gcttggttat gctcttatgt catccatgtc ctatcgctct cttctacagt caaagtgccc    180
gtcgtagget accgcagcgt ctttgagcct acatggcttc tccgtttgcg ctttgtttgg    240
gaagggggat ctatcatcgg ccaaggctac aacaaattta aagactctat cttccaggty    300
cgaaagcttg gtaccgatat cgtcatcacc cgcctcaact acatcgatga ggtcagaaag    360
ctgtcccaag acaagactcg ctcggctgag cccctcatca atgactttgc gggacagtat    420
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aaagagatgc ctgacatgaa gaatgatgaa tgggttgaag tcgacatttc ttccatcatg    600
gtcaggctca tatcacgcat ctcagccaga gtgtttctcg gtccagagca ctgccgcaac    660
caagaatggt tgacgaccac tgcagagtac agcgagagcc tgttcataac tggctttatt    720
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ccatcaggca ctcgcatcgc ggttccctct cacgcgatgc ttcaggactc agcgcgatgc   1260
ccaggccccg cgcaccacac cgagtttgat ggatttagat actcaaagat tcgctcagac   1320
tcaaactatg cacagaaata tctcttctcc atgactgatt ctagtaacat ggcggttggg   1380
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gcgatactcc ttttacaatt tgagttcaag ttgccagatg ggaaggaag accacgaaat 1500
 atcactattg atagtgacat gatacctgat ccgagagcta ggctgtgcgt taggaagcga 1560
 tcactgagag atgaatga 1578

<210> SEQ ID NO 15
 <211> LENGTH: 525
 <212> TYPE: PRT
 <213> ORGANISM: Gibberella fujikuroi

<400> SEQUENCE: 15

Met Ser Lys Ser Asn Ser Met Asn Ser Thr Ser His Glu Thr Leu Phe
 1 5 10 15
 Gln Gln Leu Val Leu Gly Leu Asp Arg Met Pro Leu Met Asp Val His
 20 25 30
 Trp Leu Ile Tyr Val Ala Phe Gly Ala Trp Leu Cys Ser Tyr Val Ile
 35 40 45
 His Val Leu Ser Ser Ser Ser Thr Val Lys Val Pro Val Val Gly Tyr
 50 55 60
 Arg Ser Val Phe Glu Pro Thr Trp Leu Leu Arg Leu Arg Phe Val Trp
 65 70 75 80
 Glu Gly Gly Ser Ile Ile Gly Gln Gly Tyr Asn Lys Phe Lys Asp Ser
 85 90 95
 Ile Phe Gln Val Arg Lys Leu Gly Thr Asp Ile Val Ile Ile Pro Pro
 100 105 110
 Asn Tyr Ile Asp Glu Val Arg Lys Leu Ser Gln Asp Lys Thr Arg Ser
 115 120 125
 Val Glu Pro Phe Ile Asn Asp Phe Ala Gly Gln Tyr Thr Arg Gly Met
 130 135 140
 Val Phe Leu Gln Ser Asp Leu Gln Asn Arg Val Ile Gln Gln Arg Leu
 145 150 155 160
 Thr Pro Lys Leu Val Ser Leu Thr Lys Val Met Lys Glu Glu Leu Asp
 165 170 175
 Tyr Ala Leu Thr Lys Glu Met Pro Asp Met Lys Asn Asp Glu Trp Val
 180 185 190
 Glu Val Asp Ile Ser Ser Ile Met Val Arg Leu Ile Ser Arg Ile Ser
 195 200 205
 Ala Arg Val Phe Leu Gly Pro Glu His Cys Arg Asn Gln Glu Trp Leu
 210 215 220
 Thr Thr Thr Ala Glu Tyr Ser Glu Ser Leu Phe Ile Thr Gly Phe Ile
 225 230 235 240
 Leu Arg Val Val Pro His Ile Leu Arg Pro Phe Ile Ala Pro Leu Leu
 245 250 255
 Pro Ser Tyr Arg Thr Leu Leu Arg Asn Val Ser Ser Gly Arg Arg Val
 260 265 270
 Ile Gly Asp Ile Ile Arg Ser Gln Gln Gly Asp Gly Asn Glu Asp Ile
 275 280 285
 Leu Ser Trp Met Arg Asp Ala Ala Thr Gly Glu Glu Lys Gln Ile Asp
 290 295 300
 Asn Ile Ala Gln Arg Met Leu Ile Leu Ser Leu Ala Ser Ile His Thr
 305 310 315 320
 Thr Ala Met Thr Met Thr His Ala Met Tyr Asp Leu Cys Ala Cys Pro
 325 330 335
 Glu Tyr Ile Glu Pro Leu Arg Asp Glu Val Lys Ser Val Val Gly Ala
 340 345 350

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Ser Gly Trp Asp Lys Thr Ala Leu Asn Arg Phe His Lys Leu Asp Ser
 355 360 365

Phe Leu Lys Glu Ser Gln Arg Phe Asn Pro Val Phe Leu Leu Thr Phe
 370 375 380

Asn Arg Ile Tyr His Gln Ser Met Thr Leu Ser Asp Gly Thr Asn Ile
 385 390 395 400

Pro Ser Gly Thr Arg Ile Ala Val Pro Ser His Ala Met Leu Gln Asp
 405 410 415

Ser Ala His Val Pro Gly Pro Thr Pro Pro Thr Glu Phe Asp Gly Phe
 420 425 430

Arg Tyr Ser Lys Ile Arg Ser Asp Ser Asn Tyr Ala Gln Lys Tyr Leu
 435 440 445

Phe Ser Met Thr Asp Ser Ser Asn Met Ala Phe Gly Tyr Gly Lys Tyr
 450 455 460

Ala Cys Pro Gly Arg Phe Tyr Ala Ser Asn Glu Met Lys Leu Thr Leu
 465 470 475 480

Ala Ile Leu Leu Leu Gln Phe Glu Phe Lys Leu Pro Asp Gly Lys Gly
 485 490 495

Arg Pro Arg Asn Ile Thr Ile Asp Ser Asp Met Ile Pro Asp Pro Arg
 500 505 510

Ala Arg Leu Cys Val Arg Lys Arg Ser Leu Arg Asp Glu
 515 520 525

<210> SEQ ID NO 16
 <211> LENGTH: 1567
 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: engineered synthetic Codon optimized Gibberella
 fujikuroi Gibberella fujikuroi Kaurene Oxidase (GFKO) gene

<400> SEQUENCE: 16

atgagtaaga gtaacagtat gaacagtaca tcccacgaaa ctttattcca acaattagta 60

ttaggtttag atagaatgcc tttgatggat gtccattggg taatctatgt tgcctttggg 120

gcttggttat gttcttactg aatacacgtc ttgtcttcat ccagtacagt taaagtacca 180

gttgtagggt atagatcagt tttcgaacct acctggttgt taagattgag atttgtttgg 240

gaaggtgggt ccatcatcgg tcaaggttac aacaaattca aggatagtat cttccaagtt 300

agaaagttag gtacagacat agtaatcatt ccacctaact acatcgatga agttagaaaa 360

ttgtctcaag acaagactag atcagtagaa ccttttatta acgatttcgc aggtcaatac 420

acaagaggta tggctctttt gcaatccgac ttacaaaaca gagttattca acaaagattg 480

acccccaaat tggtttcttt aactaaagta atgaaggaag aattggatta cgccttaact 540

aaagaaatgc ctgatatgaa gaacgacgaa tgggtcgaag ttgatatttc ttctatcatg 600

gttagattaa tatccagaat cagtgctaga gtcttcttgg gtctgaaca ttgcagaaat 660

caagaatggt tgactacaac cgcagaatat tccgaaagtt tgttatcac aggtttcatt 720

ttgagagtcg ttccacatat cttgagacct tttatcgac cattggttgc ttcatacaga 780

actttgttga gaaacgtatc cagtggtaga agagtcacgc gtgacattat cagatctcaa 840

caaggtgacg gtaacgaaga cattttatca tggatgagag atgctgcaac aggtgaagaa 900

aagcaaatcg acaacatcgc tcaaagaatg ttgatattgt ctttagcttc aatacatact 960

acagcaatga ccatgactca cgccatgtat gatttgtgtg cttgcccaga atacattgaa 1020

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cctttgagag acgaagttaa atctgtagtc ggtgcatcag gttgggataa gactgccttg 1080
aacagattcc ataaattgga ctccctttta aaagaaagtc aaagattcaa tccagttttc 1140
ttggtgacct ttaacagaat ctatcaccaa tccatgactt taagtgatgg taaaaatate 1200
ccatctggta ctagaattgc agttccttcc catgccatgt tgcaagatag tgcccacggt 1260
ccaggtccta caccacctac cgaatttgat ggtttcagat actctaagat cagatctgac 1320
tcaaaactacg ctcaaaagta cttattctca atgactgatt cttcaaacat ggcttttggg 1380
tatggtaaat acgcatgtcc aggtagattt tacgcctcta acgaaatgaa gttgacattg 1440
gctatcttgt tgttgcaatt cgagtttaaa ttgccagatg gtaaaggtag acctagaaat 1500
attaccatag attctgacat gatacctgac ccaagagcaa gattatgcgt tagaaaaaga 1560
agtttga 1567

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<210> SEQ ID NO 17

<211> LENGTH: 2607

<212> TYPE: DNA

<213> ORGANISM: *Abies grandis*

<400> SEQUENCE: 17

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atggccatgc cttcctcttc attgtcatca cagattccca ctgctgctca tcacttaact 60
gctaacgcac aatccattcc gcatttctcc acgacgctga atgctggaag cagtgctagc 120
aaacggagaa gcttgtacct acgatggggg aaaggttcaa acaagatcat tgctctgtgt 180
ggagaagggtg gtgcaacctc tgttccttat cagtctgctg aaaagaatga ttctctttct 240
tcttctacat tggtgaaacg agaatttctc ccaggatttt ggaaggatga tcttatcgat 300
tctctaactc catctcaciaa ggttgacgca tcagacgaga agcgtatcga gacattaata 360
tccgagatta agaatatggt tagatgtatg ggctatggcg aaacgaatcc ctctgcatat 420
gacactgctt gggtagcaag gattccagca gttgatggct ctgacaaccc tcactttcct 480
gagacgggtg aatggattct tcaaaatcag ttgaaagatg ggtcttgggg tgaaggattc 540
tacttcttgg catatgacag aatactggct acaactgcat gtattattac ccttaccctc 600
tggcgtactg gggagacaca agtacagaaa ggtattgaat tcttcaggac acaagctgga 660
aagatggaag atgaaactga tagtcatagg ccaagtggat ttgaaatagt atttctgca 720
atgctaaagg aagctaaaat cttaggcttg gatctgcctt acgatttggc attcctgaaa 780
caaatcatcg aaaagcggga ggctaagctt aaaaggattc ccaactgatgt tctctatgcc 840
cttccaaciaa cgttatttga ttctttggaa ggtttacaag aaatagtaga ctggcagaaa 900
ataatgaaac ttcaatccaa ggatggatca tttctcagct ctccggcacc tacagcggt 960
gtattcatgc gtacagggaa caaaaagtc ttggatttct tgaactttgt cttgaagaaa 1020
ttcgaaacc atgtgccttg tcactatccg cttgatctat ttgaaacttt gtggcggtgt 1080
gatacagttg agcggctagg tatcgatcgt catttcaaag aggagatcaa ggaagcattg 1140
gattatggtt acagccattg ggacgaaaga ggcattggat gggcgagaga gaatcctgtt 1200
cctgatattg atgatacagc catgggcctt cgaatcttga gattacatgg atacaatgta 1260
tcctcagatg ttttaaaaac atttagagat gagaatgggg agttcttttg cttcttgggt 1320
caaacacaga gaggagtac agacatgta aacgtcaatc gttgttcaca tgtttcattt 1380
ccgggagaaa cgatcatgga agaagcaaaa ctctgtaccg aaaggatctt gaggaatgct 1440
ctggaaaatg tggatgcctt tgacaaatgg gcttttaaaa agaataatcg gggagaggta 1500
gagtatgcac tcaaatatcc ctggcataag agtatgcaa ggttgaggc tagaagctat 1560

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attgaaaact atgggccaga tgatgtgtgg cttggaaaaa ctgtatatat gatgccatac 1620
atttcgaatg aaaagtattt agaactagcg aaactggact tcaataaggt gcagtctata 1680
caccaaacag agcttcaaga tcttcgaagg tggtggaat catccggttt cacggatctg 1740
aatttcactc gtgagcgtgt gacggaaata tatttctcac cggcatcctt tatctttgag 1800
cccaggtttt ctaagtgcag agaggtttat acaaaaactt ccaatttcac tgttatttta 1860
gatgatcttt atgacgccca tggatcttta gacgatctta agttgttcac agaatcagtc 1920
aaaagatggg atctatcact agtggacca atgccacaac aaatgaaaat atgttttgtg 1980
ggtttctaca atacttttaa tgatatagca aaagaaggac gtgagaggca agggcgcgat 2040
gtgctaggct acattcaaaa tgtttgaaa gtccaacttg aagcttacac gaaagaagca 2100
gaatggtctg aagctaaata tgtgccatcc ttcaatgaat acatagagaa tgcgagtgtg 2160
tcaatagcat tgggaacagt cgttctcatt agtgctcttt tcaactggga ggttcttaca 2220
gatgaagtac tctccaaat tgatcgcgaa tctagatttc ttcaactcat gggcttaaca 2280
ggggttttg tgaatgacac caaaaactat caggcagaga gaggtcaagg tgaggtggct 2340
tctgccatac aatgttatat gaaggacat cctaaaatct ctgaagaaga agctctacaa 2400
catgtctata gtgtcatgga aaatgccctc gaagagttga ataggaggtt tgtgaataac 2460
aaaaataccg atatttaca aagactggtt tttgaaactg caagaataat gcaactcttt 2520
tatatgcaag gggatggttt gacactatca catgatatgg aaattaaaga gcatgtcaaa 2580
aattgcctct tccaaccagt tgcctag 2607

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<210> SEQ ID NO 18
<211> LENGTH: 868
<212> TYPE: PRT
<213> ORGANISM: Abies grandis

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<400> SEQUENCE: 18

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Met Ala Met Pro Ser Ser Ser Leu Ser Ser Gln Ile Pro Thr Ala Ala
1 5 10 15
His His Leu Thr Ala Asn Ala Gln Ser Ile Pro His Phe Ser Thr Thr
20 25 30
Leu Asn Ala Gly Ser Ser Ala Ser Lys Arg Arg Ser Leu Tyr Leu Arg
35 40 45
Trp Gly Lys Gly Ser Asn Lys Ile Ile Ala Cys Val Gly Glu Gly Gly
50 55 60
Ala Thr Ser Val Pro Tyr Gln Ser Ala Glu Lys Asn Asp Ser Leu Ser
65 70 75 80
Ser Ser Thr Leu Val Lys Arg Glu Phe Pro Pro Gly Phe Trp Lys Asp
85 90 95
Asp Leu Ile Asp Ser Leu Thr Ser Ser His Lys Val Ala Ala Ser Asp
100 105 110
Glu Lys Arg Ile Glu Thr Leu Ile Ser Glu Ile Lys Asn Met Phe Arg
115 120 125
Cys Met Gly Tyr Gly Glu Thr Asn Pro Ser Ala Tyr Asp Thr Ala Trp
130 135 140
Val Ala Arg Ile Pro Ala Val Asp Gly Ser Asp Asn Pro His Phe Pro
145 150 155 160
Glu Thr Val Glu Trp Ile Leu Gln Asn Gln Leu Lys Asp Gly Ser Trp
165 170 175
Gly Glu Gly Phe Tyr Phe Leu Ala Tyr Asp Arg Ile Leu Ala Thr Leu

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180				185				190							
Ala	Cys	Ile	Ile	Thr	Leu	Thr	Leu	Trp	Arg	Thr	Gly	Glu	Thr	Gln	Val
		195					200					205			
Gln	Lys	Gly	Ile	Glu	Phe	Phe	Arg	Thr	Gln	Ala	Gly	Lys	Met	Glu	Asp
	210					215					220				
Glu	Ala	Asp	Ser	His	Arg	Pro	Ser	Gly	Phe	Glu	Ile	Val	Phe	Pro	Ala
225					230					235					240
Met	Leu	Lys	Glu	Ala	Lys	Ile	Leu	Gly	Leu	Asp	Leu	Pro	Tyr	Asp	Leu
			245						250					255	
Pro	Phe	Leu	Lys	Gln	Ile	Ile	Glu	Lys	Arg	Glu	Ala	Lys	Leu	Lys	Arg
		260						265						270	
Ile	Pro	Thr	Asp	Val	Leu	Tyr	Ala	Leu	Pro	Thr	Thr	Leu	Leu	Tyr	Ser
		275					280					285			
Leu	Glu	Gly	Leu	Gln	Glu	Ile	Val	Asp	Trp	Gln	Lys	Ile	Met	Lys	Leu
	290					295					300				
Gln	Ser	Lys	Asp	Gly	Ser	Phe	Leu	Ser	Ser	Pro	Ala	Ser	Thr	Ala	Ala
305					310					315					320
Val	Phe	Met	Arg	Thr	Gly	Asn	Lys	Lys	Cys	Leu	Asp	Phe	Leu	Asn	Phe
				325					330					335	
Val	Leu	Lys	Lys	Phe	Gly	Asn	His	Val	Pro	Cys	His	Tyr	Pro	Leu	Asp
		340						345					350		
Leu	Phe	Glu	Arg	Leu	Trp	Ala	Val	Asp	Thr	Val	Glu	Arg	Leu	Gly	Ile
		355					360					365			
Asp	Arg	His	Phe	Lys	Glu	Glu	Ile	Lys	Glu	Ala	Leu	Asp	Tyr	Val	Tyr
	370					375					380				
Ser	His	Trp	Asp	Glu	Arg	Gly	Ile	Gly	Trp	Ala	Arg	Glu	Asn	Pro	Val
385					390					395					400
Pro	Asp	Ile	Asp	Asp	Thr	Ala	Met	Gly	Leu	Arg	Ile	Leu	Arg	Leu	His
			405						410					415	
Gly	Tyr	Asn	Val	Ser	Ser	Asp	Val	Leu	Lys	Thr	Phe	Arg	Asp	Glu	Asn
		420						425					430		
Gly	Glu	Phe	Phe	Cys	Phe	Leu	Gly	Gln	Thr	Gln	Arg	Gly	Val	Thr	Asp
		435					440					445			
Met	Leu	Asn	Val	Asn	Arg	Cys	Ser	His	Val	Ser	Phe	Pro	Gly	Glu	Thr
	450					455					460				
Ile	Met	Glu	Glu	Ala	Lys	Leu	Cys	Thr	Glu	Arg	Tyr	Leu	Arg	Asn	Ala
465					470					475					480
Leu	Glu	Asn	Val	Asp	Ala	Phe	Asp	Lys	Trp	Ala	Phe	Lys	Lys	Asn	Ile
			485						490					495	
Arg	Gly	Glu	Val	Glu	Tyr	Ala	Leu	Lys	Tyr	Pro	Trp	His	Lys	Ser	Met
			500					505					510		
Pro	Arg	Leu	Glu	Ala	Arg	Ser	Tyr	Ile	Glu	Asn	Tyr	Gly	Pro	Asp	Asp
		515					520					525			
Val	Trp	Leu	Gly	Lys	Thr	Val	Tyr	Met	Met	Pro	Tyr	Ile	Ser	Asn	Glu
	530					535						540			
Lys	Tyr	Leu	Glu	Leu	Ala	Lys	Leu	Asp	Phe	Asn	Lys	Val	Gln	Ser	Ile
545					550					555					560
His	Gln	Thr	Glu	Leu	Gln	Asp	Leu	Arg	Arg	Trp	Trp	Lys	Ser	Ser	Gly
			565						570					575	
Phe	Thr	Asp	Leu	Asn	Phe	Thr	Arg	Glu	Arg	Val	Thr	Glu	Ile	Tyr	Phe
			580						585				590		
Ser	Pro	Ala	Ser	Phe	Ile	Phe	Glu	Pro	Glu	Phe	Ser	Lys	Cys	Arg	Glu
		595					600						605		

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Val Tyr Thr Lys Thr Ser Asn Phe Thr Val Ile Leu Asp Asp Leu Tyr
 610 615 620

Asp Ala His Gly Ser Leu Asp Asp Leu Lys Leu Phe Thr Glu Ser Val
 625 630 635 640

Lys Arg Trp Asp Leu Ser Leu Val Asp Gln Met Pro Gln Gln Met Lys
 645 650 655

Ile Cys Phe Val Gly Phe Tyr Asn Thr Phe Asn Asp Ile Ala Lys Glu
 660 665 670

Gly Arg Glu Arg Gln Gly Arg Asp Val Leu Gly Tyr Ile Gln Asn Val
 675 680 685

Trp Lys Val Gln Leu Glu Ala Tyr Thr Lys Glu Ala Glu Trp Ser Glu
 690 695 700

Ala Lys Tyr Val Pro Ser Phe Asn Glu Tyr Ile Glu Asn Ala Ser Val
 705 710 715 720

Ser Ile Ala Leu Gly Thr Val Val Leu Ile Ser Ala Leu Phe Thr Gly
 725 730 735

Glu Val Leu Thr Asp Glu Val Leu Ser Lys Ile Asp Arg Glu Ser Arg
 740 745 750

Phe Leu Gln Leu Met Gly Leu Thr Gly Arg Leu Val Asn Asp Thr Lys
 755 760 765

Thr Tyr Gln Ala Glu Arg Gly Gln Gly Glu Val Ala Ser Ala Ile Gln
 770 775 780

Cys Tyr Met Lys Asp His Pro Lys Ile Ser Glu Glu Glu Ala Leu Gln
 785 790 795 800

His Val Tyr Ser Val Met Glu Asn Ala Leu Glu Glu Leu Asn Arg Glu
 805 810 815

Phe Val Asn Asn Lys Ile Pro Asp Ile Tyr Lys Arg Leu Val Phe Glu
 820 825 830

Thr Ala Arg Ile Met Gln Leu Phe Tyr Met Gln Gly Asp Gly Leu Thr
 835 840 845

Leu Ser His Asp Met Glu Ile Lys Glu His Val Lys Asn Cys Leu Phe
 850 855 860

Gln Pro Val Ala
 865

<210> SEQ ID NO 19
 <211> LENGTH: 2607
 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: engineered sequence
 <400> SEQUENCE: 19

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 gcaaacgcac aaagtattcc acatttttct actacactta atgctggatc tagtgcttct 120
 aagaggagat cattgtattt gagatgggga aaaggatcca acaagattat tgcattgctg 180
 ggagaaggag gtgcaacatc agttccttac caatctgctg agaagaatga ttctttaagt 240
 tcttcaacac ttgtgaaaag ggagtttcca cctggttttt ggaagaatga tctcattgat 300
 tctttaactt ctcccaataa agtggtctgca tccgatgaaa aaaggattga gactctcatt 360
 tctgaaatta agaacatggt tagatgtatg ggttacggag aaactaacc tctgcttac 420
 gatacagctt gggttgctag gattccagct gtggatggta gtgataaacc acattttcct 480
 gagactgttg aatggattct tcagaatcag ctcaaagatg gttcttgggg agaaggattc 540

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tatttcttag cttacgatag aattttggca actttggcct gcattattac tttgacactt 600
tggagaactg gtgaaacaca agttcagaag ggtattgaat ttttcaggac tcaagcagga 660
aagatggagg atgaggctga tagtcacaga ccttcagggt tcgagattgt gtttccagca 720
atggtgaaag aggctaagat tcttggattg gatcttcctt acgatttgcc atttctcaag 780
caaattattg agaaaagaga agctaagctc aaaaggattc ctacagatgt tctctacgca 840
ttaccaacaa ctcttttcta ttctttgtaa ggacttcaag aaattgttga ttggcaaaaag 900
attatgaaac tccaaagtaa ggatggatct tttctctcat ctctgcttc tactgctgct 960
gtttttatga ggacaggtaa caagaagtgt ttagatttct taaatttcgt gctcaaaaag 1020
tttgaaatc atgttccatg ccactatcct cttgatttat ttgaaagact ttgggctggt 1080
gatcacagtg agaggcttgg tattgatagg cattttaaag aagaaattaa ggaggcattg 1140
gattatgttt actctcattg ggatgagaga ggaattggat gggctagaga aaacctggt 1200
cctgatattg atgatacagc aatgggtcct agaattttaa gattgcatgg atacaatgtt 1260
tcttcagatg ttttaaaaac atttagagat gagaatggag agttctctcg ctttttaggt 1320
cagacacaaa ggggagttac agatatgttg aatgttaaca gatgttctca tgttagtttc 1380
cctggtgaga ctattatgga ggaagctaag ttgtgcacag agagatatct tagaaatgca 1440
ttggagaatg ttgatgcttt cgataaatgg gcattcaaaa agaatttag ggggaagtg 1500
gaatatgctc tcaagtacc c atggcataag tctatgccta ggttgagggc tagatcatat 1560
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atttcaaatg aaaaatacct tgaactcgtc aagctcgatt ttaataaggt tcagtctatt 1680
caccaaaactg agttgcagga ttttaaggagg tgggtgaaat cttcaggatt cactgatctt 1740
aattttacta gagagagagt tactgagatt tacttctcac ctgctagttt tattttcgaa 1800
ccagagtctc caaaatgtag agaggtttat acaaaaacta gtaatttcac agttattttg 1860
gatgatttgt acgatgctca cggtagcttc gatgatctta aactttttac agaactcagtt 1920
aaaagatggg atttgcatt agttgatcaa atgccacaac aaatgaagat ttgttttggt 1980
ggattttaca atacttttaa tgatattgct aaagagggtg gggagagaca aggttagagat 2040
gttcttggat atattcagaa cgtttgaaa gtgcagttag aggtttatc aaaagaagca 2100
gagtggctcg aggcaaaagta tgtgccatct ttcaatgagt acattgaaa cgcactctgt 2160
agtattgctc tcggtactgt tgtgcttatt tcagctttat ttacaggaga ggtgcttact 2220
gatgaagtgt tgtccaaaat tgataggag agtagatttc ttcaacttat ggttcttaca 2280
ggtaggcttg ttaatgatac aaagacttat caagctgaaa ggggtcaagg agaagtgtct 2340
tctgctatct aatgttatat gaaggatcat cctaaaattt ctgaagaaga agcattgcaa 2400
catgtttatt cagtgatgga aaacgcactc gaagaattaa ataggaggtt cgtaacaac 2460
aaaaatccag atatttataa aagacttggt tttgaaactg ctagaattat gcagctcttt 2520
tacatgcaag gtgatggatt aactttgtcc catgatatgg aaattaagga gcacgttaaa 2580
aattgtttgt tccaacctgt tgcataa 2607

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<210> SEQ ID NO 20

<211> LENGTH: 658

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: engineered sequence

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<400> SEQUENCE: 20

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gagctcagtt tatcattatc aatactcgcc atttcaaaga atacgtaaat aattaatagt    60
agtgattttc ctaactttat ttagtcaaaa aattagcctt ttaattctgc tgtaaccgt    120
acatgcccaa aatagggggc gggttacaca gaatatataa catcgtaggt gtctgggtga    180
acagtttatt cctggcatcc actaaatata atggagcccg ctttttaagc tggcatccag    240
aaaaaaaaag aatcccagca ccaaaatatt gttttcttca ccaaccatca gttcataggt    300
ccattctctt agcgcaacta cagagaacag gggcacaac aggcacacac cgggcacaac    360
ctcaatggag tgatgcaacc tgcctggagt aaatgatgac acaaggcaat tgaccacgc    420
atgtatctat ctcaattttc tacaccttct attaccttct gctctctctg atttgaaaa    480
agctgaaaaa aaaggttgaa accagttccc tgaaattatt ccctacttg actaataagt    540
atataaagac ggtaggtatt gattgtaatt ctgtaaatct atttcttaa cttcttaaat    600
tctactttta tagttagtct ttttttagt tttaaacac cagaacttag tttcgacg    658

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<210> SEQ ID NO 21
<211> LENGTH: 666
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: engineered sequence

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<400> SEQUENCE: 21

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gctatcaagt ataaatagac ctgcaattat taatcttttg tttctctgctc attgttcteg    600
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 t 421

The invention claimed is:
 1. A genetically modified yeast for the enhanced expression of terpenes produced by a method comprising:
 combining yeast with a chemical mutagenesis agent to induce mutations in the yeast to generate chemically mutated yeast;
 selecting chemically mutated yeast which grows in the presence of nystatin, squalestatin and cholesterol, followed by selecting for sterol dependent growth in the presence of squalestatin;
 subjecting the sterol dependent growth yeast to an erg9 knockout mutation, to thereby produce sterol dependent growth/erg9 knockout mutation yeast cell lines; and
 inserting an expression vector into the sterol dependent growth/erg9 knockout mutation yeast cells wherein the expression vector expresses a gene for mutant avian

50 farnesyl diphosphate synthase, to thereby produce the genetically modified yeast having a dispensable sterol biosynthetic pathway and genetically modified with a non-naturally occurring prenyltransferase to thereby alter prenyl diphosphate levels and genetically modified to express a terpene synthase directed to diterpene production.
 2. The genetically modified yeast of claim 1, wherein the yeast both has an erg9 knockout and has sterol uptake enhancement (SUE) and the yeast can grow in the presence of squalestatin and nystatin and are dependent on exogenous sterol for growth.
 3. The genetically modified yeast of claim 1, wherein the yeast is selected from the group consisting of *Candida albicans* and *Saccharomyces cerevisiae*.
 4. A method for generating terpene producing yeast cell lines, the method comprising:

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combining yeast with a chemical mutagenesis agent to induce mutations in the yeast to generate chemically mutated yeast;

selecting chemically mutated yeast which grows in the presence of nystatin, squalestatin and cholesterol, followed by selecting for sterol dependent growth in the presence of squalestatin;

subjecting the sterol dependent growth yeast to an *erg9* knockout mutation, to thereby produce sterol dependent growth/*erg9* knockout mutation yeast cell lines; and

inserting an expression vector into the sterol dependent growth/*erg9* knockout mutation yeast cells wherein the expression vector expresses a gene for mutant avian farnesyl diphosphate synthase.

5. The method of claim 4, wherein subjecting the sterol dependent growth yeast to an *erg9* knockout mutation comprises inserting a foreign gene sequence into the sterol dependent growth yeast at the location of *erg9* to effect gene replacement, thereby generating the *erg9* knockout mutation.

6. The method of claim 5, wherein the foreign gene sequence confers chemical resistance to a selected chemical thereby allowing the sterol dependent growth/*erg9* knockout mutation yeast to grow in the presence of the chemical.

7. The method of claim 4, wherein the yeast is selected from the group consisting of *Candida albicans* and *Saccharomyces cerevisiae*.

8. The genetically modified yeast of claim 1, wherein the terpene synthase is kaurene synthase.

9. The genetically modified yeast of claim 1, wherein the prenyltransferase and the terpene synthase are targeted to the cytoplasm of the genetically modified yeast.

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10. The generally modified yeast of claim 1, wherein the dispensable sterol biosynthetic pathway comprises a functional mevalonate pathway not coupled to sterol metabolism.

11. The genetically modified yeast of claim 1, wherein the non-naturally occurring prenyltransferase catalyzes the conversion of dimethylallyl pyrophosphate (DMAPP) and isopentenyl diphosphate (IPP) to yield GGPP.

12. The genetically modified yeast of claim 11, wherein the terpene synthase, expressed for diterpene production by genetic modification, is a heterologous diterpene synthase that converts available geranylgeranyl diphosphate (GGPP) to the production of diterpenes.

13. The genetically modified yeast of claim 1, wherein the terpene synthase, expressed for diterpene production by genetic modification, is a heterologous diterpene synthase that converts available geranylgeranyl diphosphate (GGPP) to the production of diterpenes.

14. The genetically modified yeast of claim 1, wherein subjecting the sterol dependent growth yeast to an *erg9* knockout mutation comprises inserting a foreign gene sequence into the sterol dependent growth yeast at the location of *erg9* to effect gene replacement, thereby generating the *erg9* knockout mutation.

15. The genetically modified yeast of claim 14, wherein the foreign gene sequence confers chemical resistance to a selected chemical thereby allowing the sterol dependent growth/*erg9* knockout mutation yeast to grow in the presence of the chemical.

16. The genetically modified yeast of claim 1, wherein the sterol is ergosterol.

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