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Method and System for Diterpene Production Platforms in Yeast

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

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     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
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(57)   **ABSTRACT**

A method is provided for modifying yeast to express mutant avian farnesyl disphospate 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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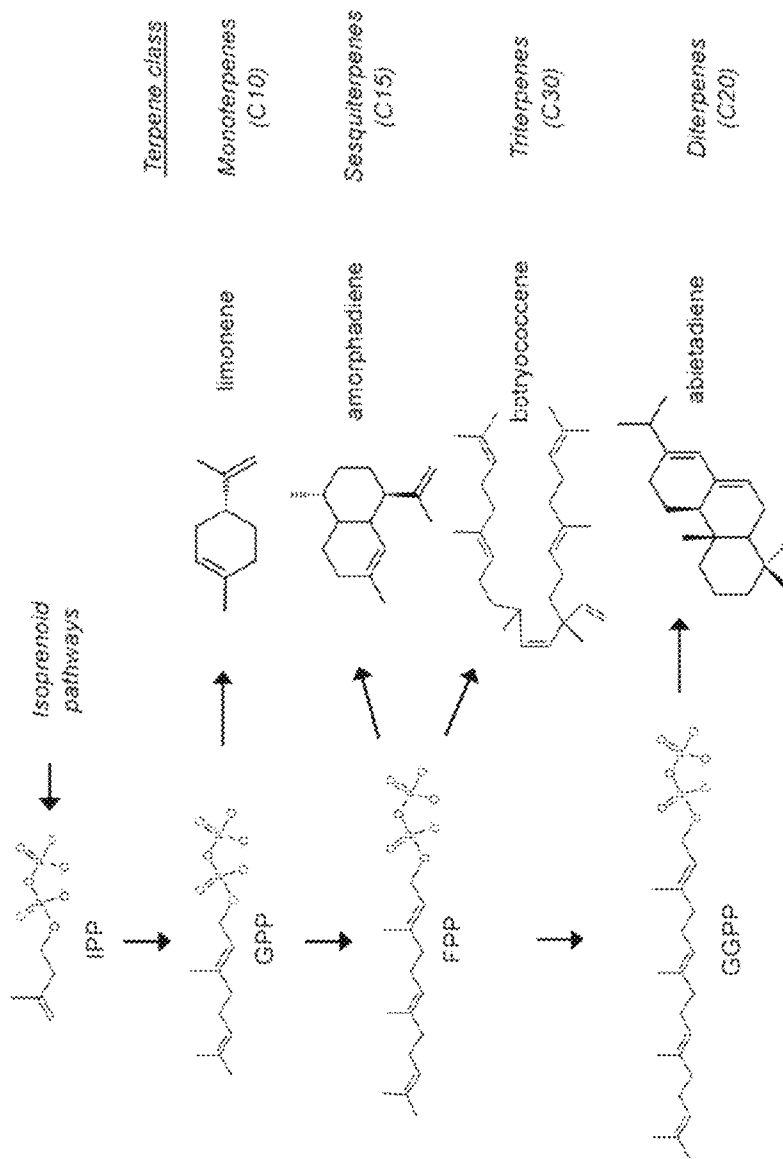Figure 1

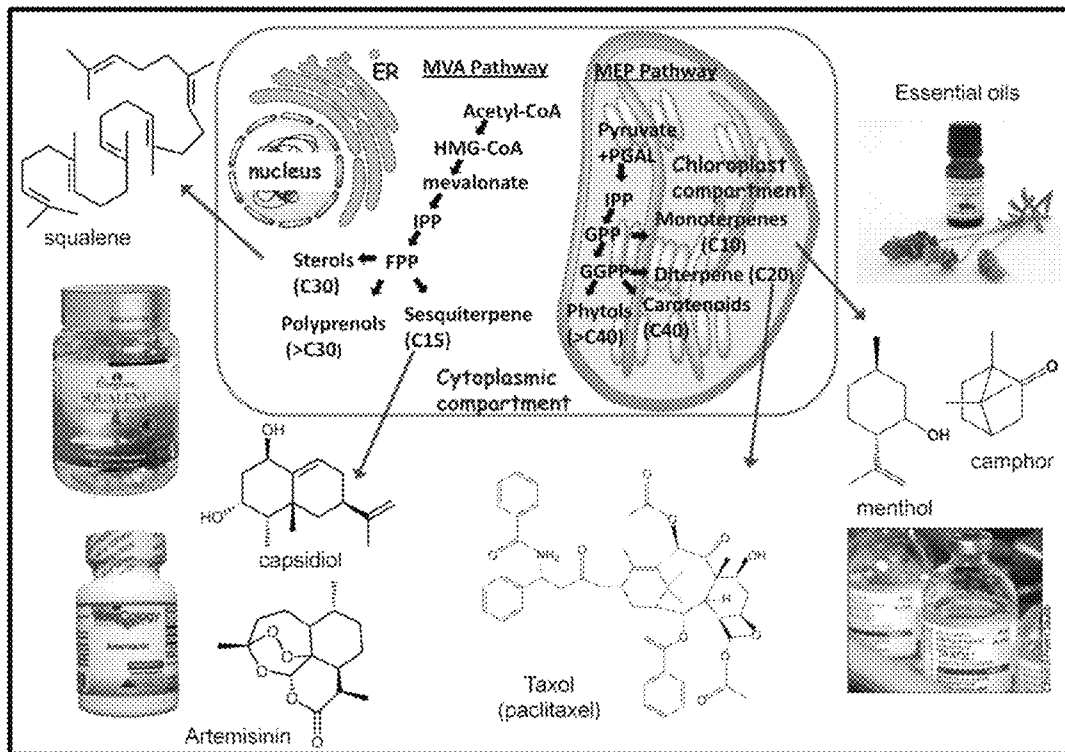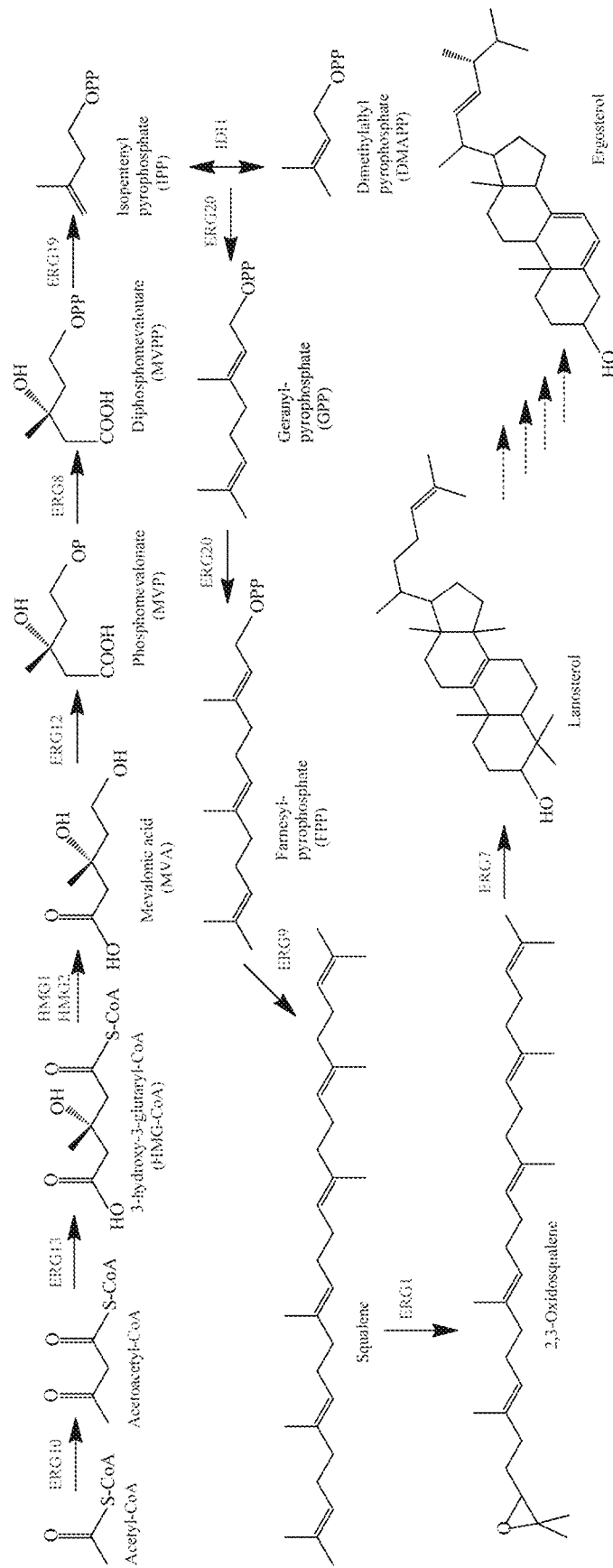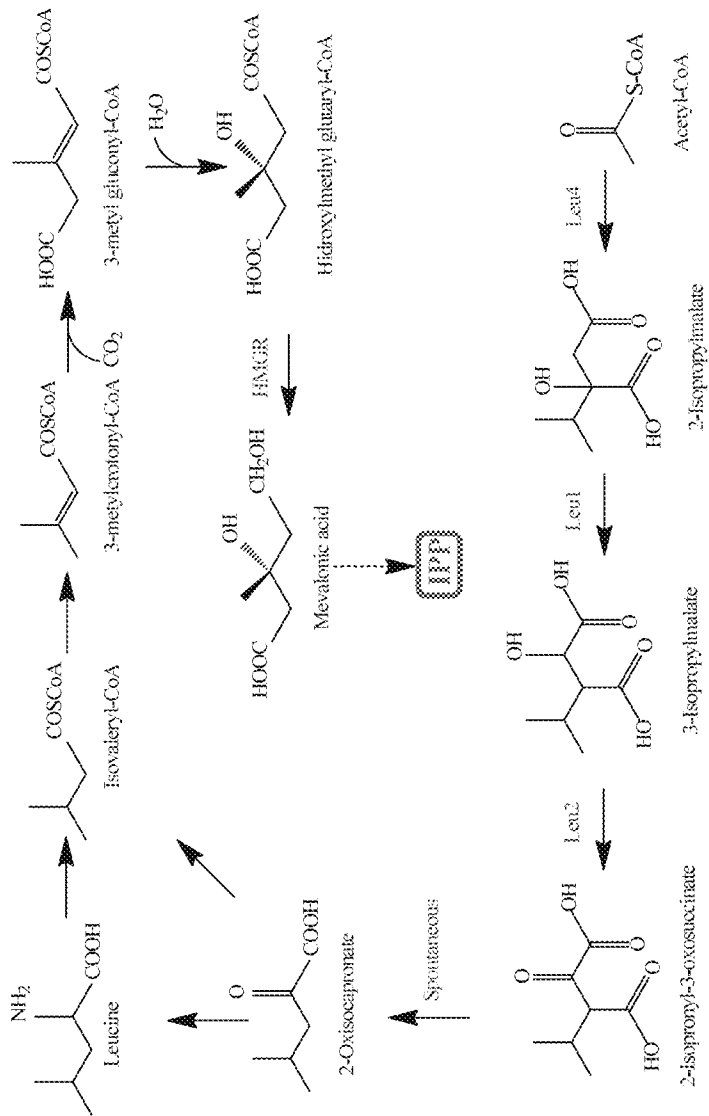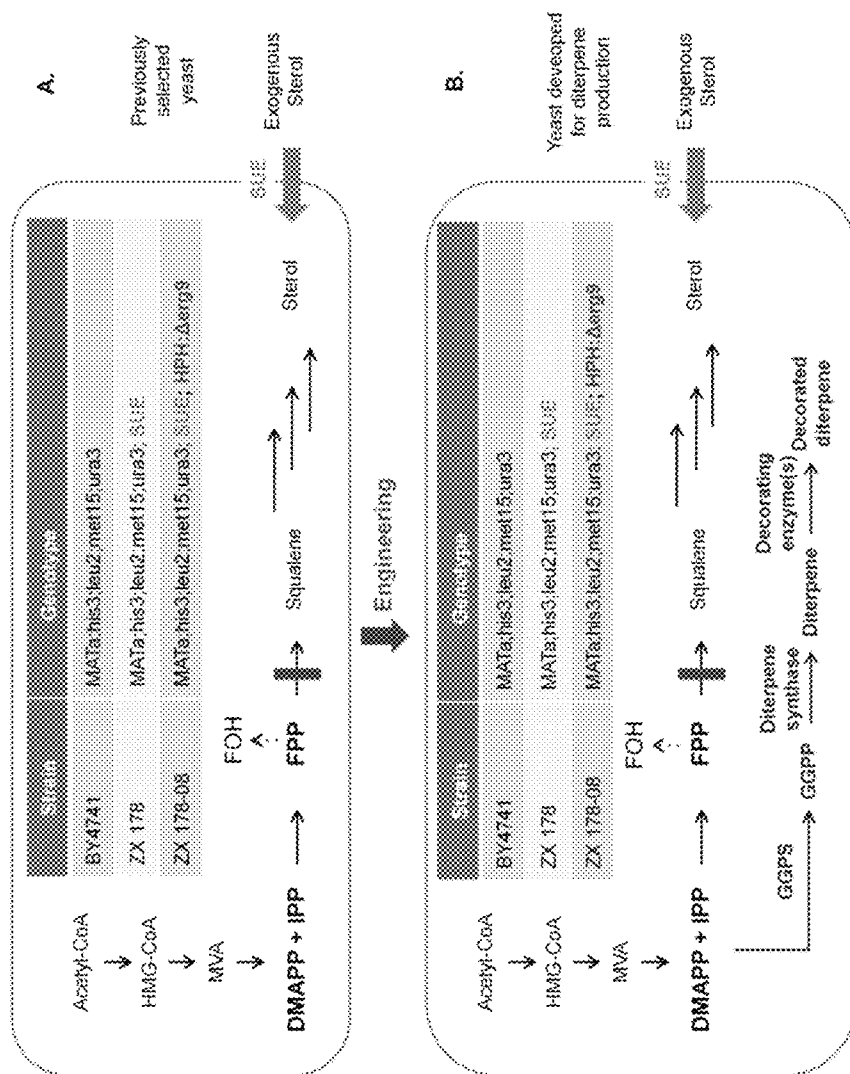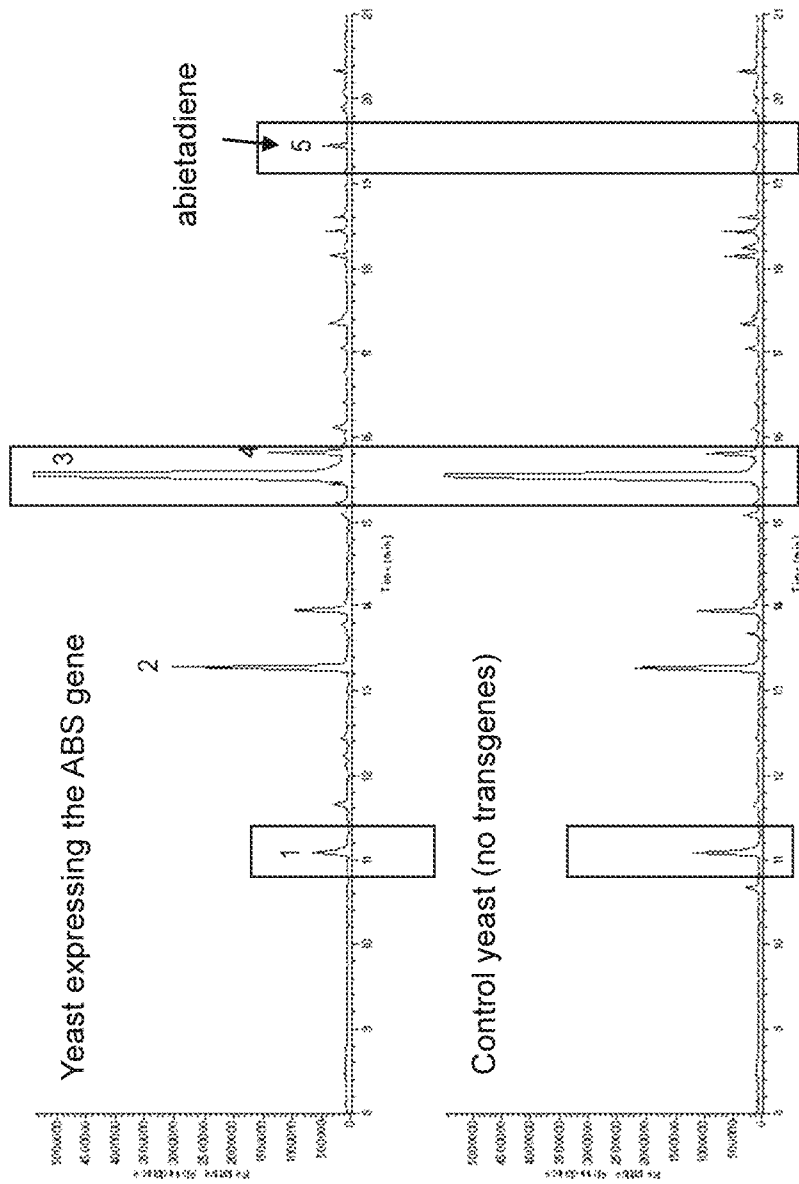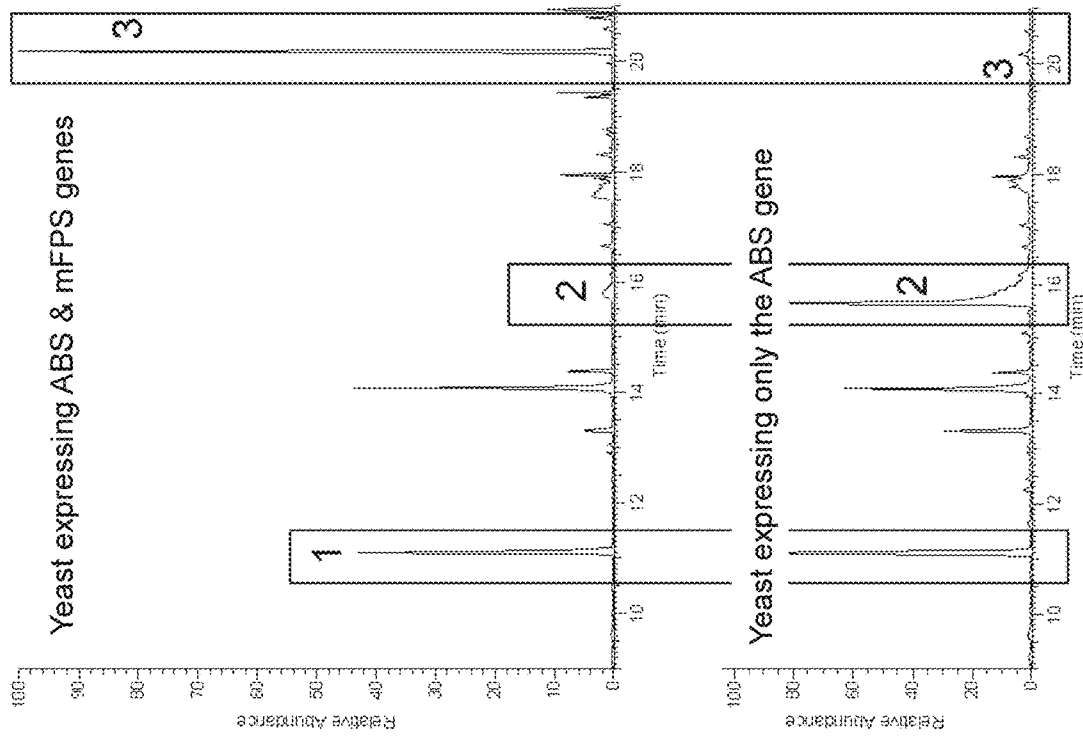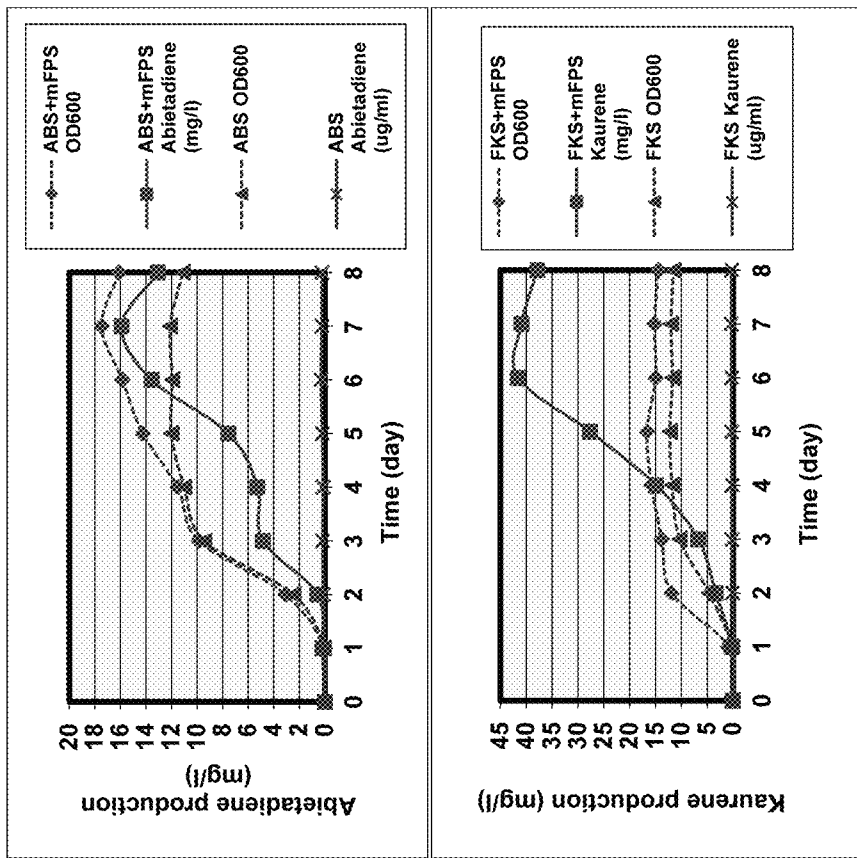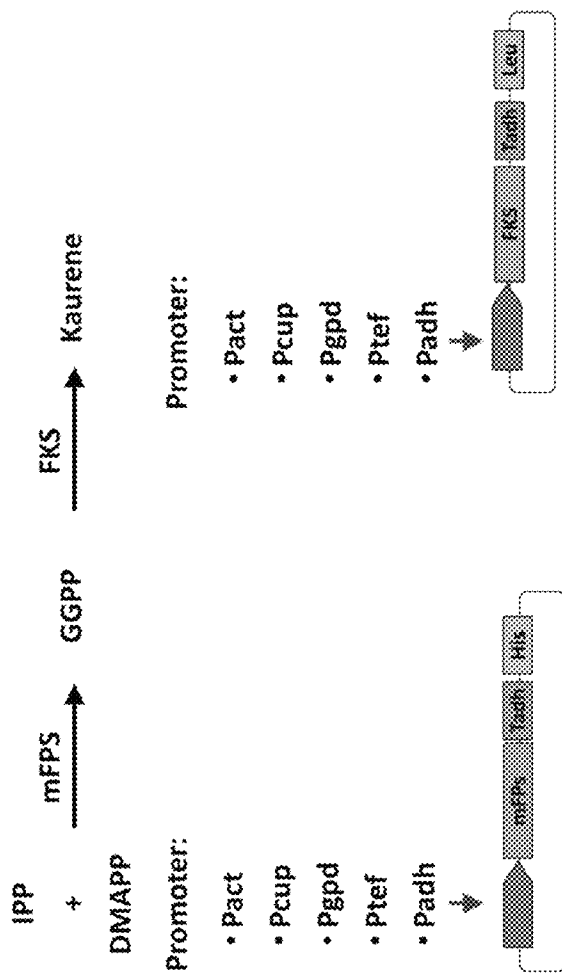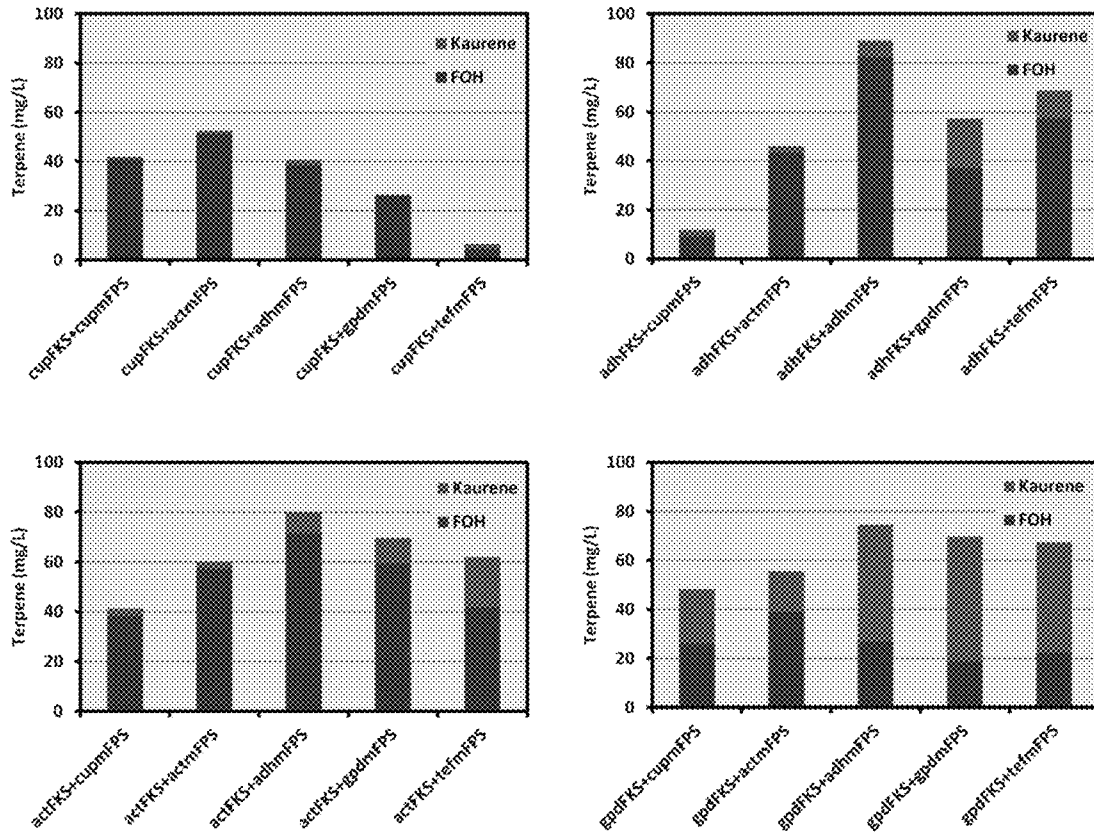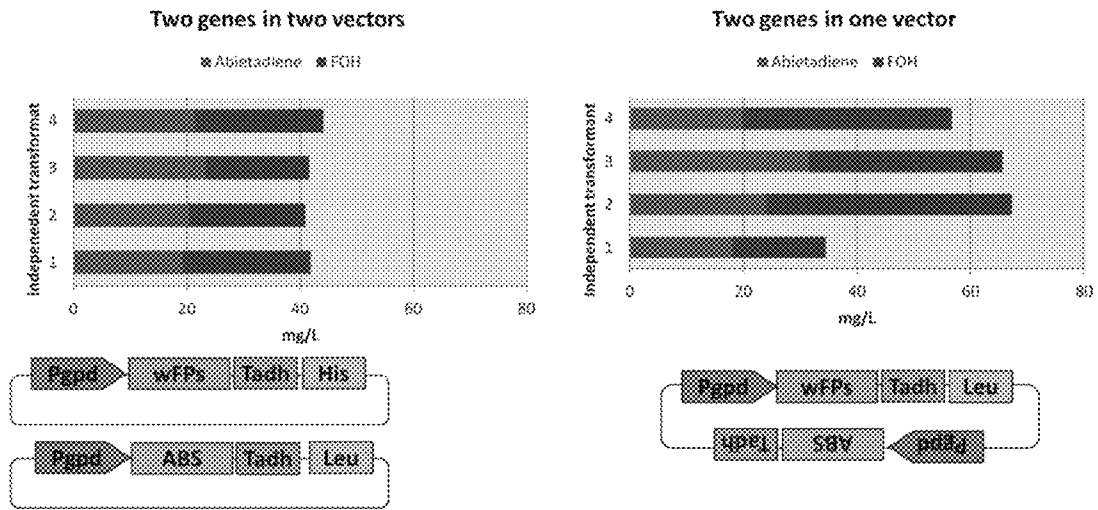**Figure 2**

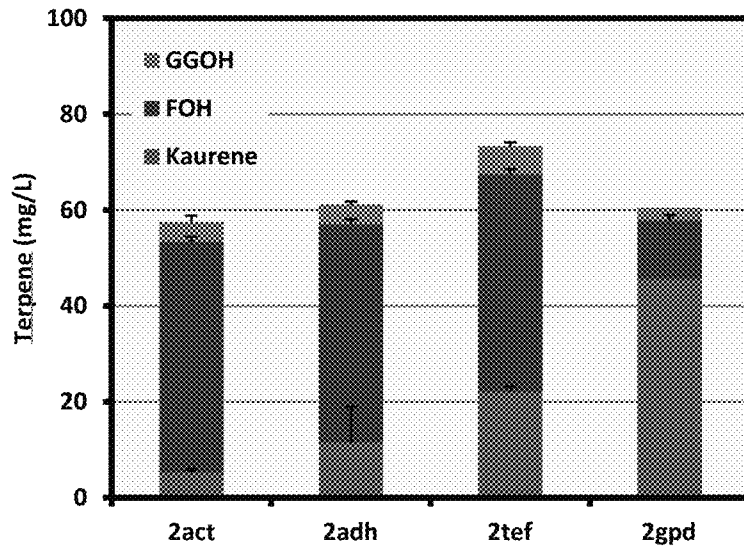**Figure 3**

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8

Figure 9

Figure 10

Figure 11

Figure 12

Figure 13
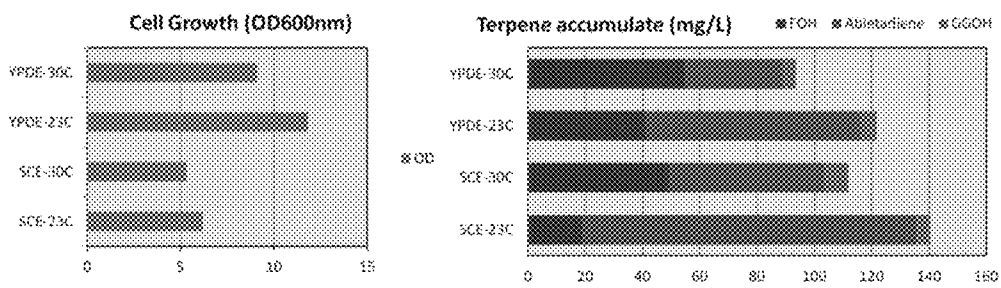
GFKO: Fungal Kaurene oxidase
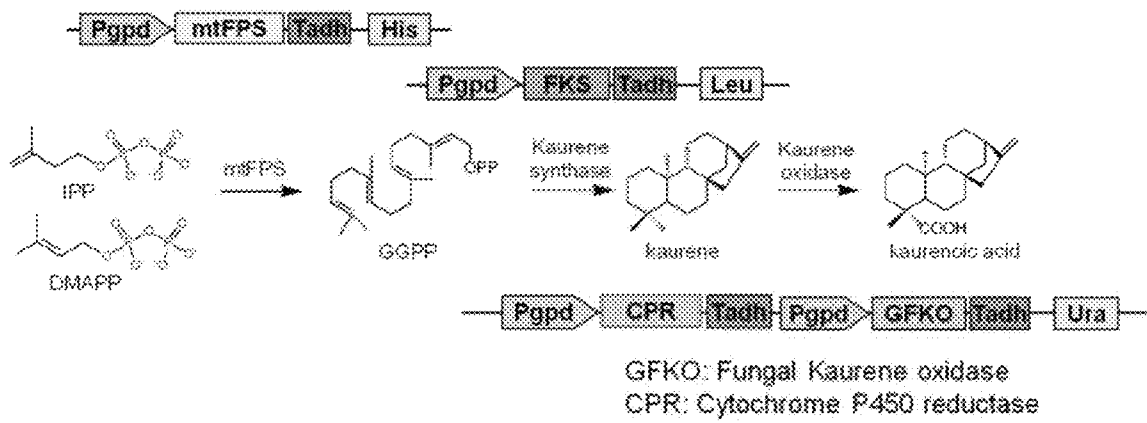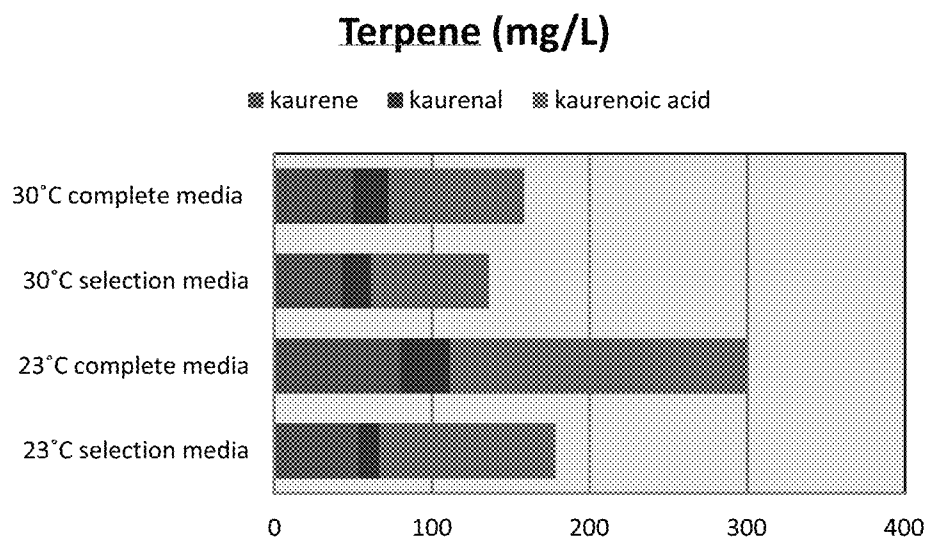CPR: Cytochrome P450 reductase

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 disphospate 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 live 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 regent (Barkovich & Liao, 2001). Triterpenes (C30) include the brassinosteroids, phytosterols important for lipid membrane composition, and components of surface waxes, such as oleanolic aid 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 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-3methyglutaryl-CoA (HMG-CoA).

TABLE 1

Biological activities and commercial applications of typical terpenoids

Class	Biologic activities	Commercial applications	Examples
Monoter-penoids	Signal molecules and used as defense mechanisms against pathogens	Flavors, fragrances, cleaning products, anticancer, antibacterial, antioxidant, essential oil, bionief	Limonence, menthol, camphor, linalool
Sesquiter-penoids	Antibiotic, antitumor, antiviral, immuno-suppressive, and hormonal activities, defensive agents or pheromones	Flavors, fragrances, pharmaceuticals (antibacterial, antifungal), insecticides, biofuels	Nootkatone, artemisinin, patchoulol, nerolidol, farnesol, capsidol, farnesene, bisabolene
Diter-penoids	Hormonal activities, growth regulator, antitumor, antimicrobial and anti-inflammatory properties	Anticancer agents, feedstock for industrial chemical applications	Gibberellins, phytol, taxot, kaurerte, abietadiene, kaurenoic acid, abictic acid
Triter-penoids	Membrane component, steroid hormones	Biologic markers, biofuel, skin moisturizers in cosmetics, immunologic adjuvant in vaccines.	Sterols, hopanoids, squalene, botryococcene.
Tetrater-penoids	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 ERGS, 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 syntahases 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 prenyltranferase 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 (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 classes of terpenes, and more so for the generation of stereochemically pure forms of terpenes (Martin et al., 2003; Wu et al., 2006;

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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 anti-oxidant activity of diterpenes has also led to their use in human nutraceuticals and medical applications (Cardenas et al., 2011). Perhaps the most widely recognized diterpene is taxol, used very successfully and extensively for the treatment of a variety of cancers (Wall 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 tackifers (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 disphospate synthase and preferably mutant avian farnesyl disphospate synthase. The modification advantageously is provided by an expression vector encoding mutant avian farnesyl disphospate 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+.

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The mutant avian farnesyl disphospate synthase (mtFPS) converts dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP) to teganylgeranyldiphosphate (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 nysatin, 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 growthlerg9 knockout mutation yeast cell lines. An expression vector is inserted into the ergosterol dependent growthlerg9 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 erogsterol 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 abietadine 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

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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 about 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 overexpressing 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 (lg/L) and 40 mg/L ergosterol. Cholesterol and ergostrol stocks were 10 mg/mL in 50% Triton X-100, 50% ethanol and kept at −20° C. Selection media was prepared similarly except without supplementing the media with the indicated reagent based on the yeast auxotrophic makers. All solid media plates were prepared with 2% Bacto-Agar.

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 CALI7 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 $\frac{1}{10}$ the original volume. An aliquot of the organic phase (1 pl) 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×0.25 mm×0.25 pm 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 Notl 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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gagttcgtgg ggttcttccc gcagatcgtc cgcgatctga ccgaggacgg catcggacac       180

ccggaggtgg gcgacgctgt ggcgcggctg aaggaggtgc tgcaatacaa cgctcccggt       240

gggaaatgca atcgtgggct gacggtggtg gctgcgtacc gggagctgtc ggggccgggg       300

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Val Val Val Glu Arg Glu Arg Glu Glu Phe Val Gly Phe Phe Pro Gln
            35                  40                  45

Ile Val Arg Asp Leu Thr Glu Asp Gly Ile Gly His Pro Glu Val Gly
        50                  55                  60

Asp Ala Val Ala Arg Leu Lys Glu Val Leu Gln Tyr Asn Ala Pro Gly
65                  70                  75                  80

Gly Lys Cys Asn Arg Gly Leu Thr Val Val Ala Ala Tyr Arg Glu Leu
                85                  90                  95

Ser Gly Pro Gly Gln Lys Asp Ala Glu Ser Leu Arg Cys Ala Leu Ala
            100                 105                 110

Val Gly Trp Cys Ile Glu Leu Phe Gln Ala Phe Phe Leu Val Ala Asp
            115                 120                 125

Asp Ile Met Asp Gln Ser Leu Thr Arg Arg Gly Gln Leu Cys Trp Tyr
        130                 135                 140

Lys Lys Glu Gly Val Gly Leu Asp Ala Ile Asn Asp Ser Phe Leu Leu
145                 150                 155                 160

Glu Ser Ser Val Tyr Arg Val Leu Lys Lys Tyr Cys Arg Gln Arg Pro
                165                 170                 175

Tyr Tyr Val His Leu Leu Glu Leu Phe Leu Gln Thr Ala Tyr Gln Thr
                180                 185                 190

Glu Leu Gly Gln Met Leu Asp Leu Ile Thr Ala Pro Val Ser Lys Val
            195                 200                 205

Asp Leu Ser His Phe Ser Glu Glu Arg Tyr Lys Ala Ile Val Lys Tyr
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Lys Thr Ala Phe Tyr Ser Phe Tyr Leu Pro Val Ala Ala Ala Met Tyr
225                 230                 235                 240

Met Val Gly Ile Asp Ser Lys Glu Glu His Glu Asn Ala Lys Ala Ile
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Leu Leu Glu Met Gly Glu Tyr Phe Gln Ile Gln Asp Asp Tyr Leu Asp
            260                 265                 270

Cys Phe Gly Asp Pro Ala Leu Thr Gly Lys Val Gly Thr Asp Ile Gln
        275                 280                 285

Asp Asn Lys Cys Ser Trp Leu Val Val Gln Cys Leu Gln Arg Val Thr
    290                 295                 300

Pro Glu Gln Arg Gln Leu Leu Glu Asp Asn Tyr Gly Arg Lys Glu Pro
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Glu Lys Val Ala Lys Val Lys Glu Leu Tyr Glu Ala Val Gly Met Arg
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Ala Ala Phe Gln Gln Tyr Glu Glu Ser Ser Tyr Arg Arg Leu Gln Glu
            340                 345                 350

Leu Ile Glu Lys His Ser Asn Arg Leu Pro Lys Glu Ile Phe Leu Gly
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Ala Leu Arg Asn Leu Ser Pro Val Val Val Glu Arg Glu Arg Glu Glu
        35   40   45

Phe Val Gly Phe Phe Pro Gln Ile Val Arg Asp Leu Thr Glu Asp Gly
    50   55   60

Ile Gly His Pro Glu Val Gly Asp Ala Val Ala Arg Leu Lys Glu Val
65   70   75   80

Leu Gln Tyr Asn Ala Pro Gly Gly Lys Cys Asn Arg Gly Leu Thr Val
                85   90   95

Val Ala Ala Tyr Arg Glu Leu Ser Gly Pro Gly Gln Lys Asp Ala Glu
                100   105   110

Ser Leu Arg Cys Ala Leu Ala Val Gly Trp Cys Ile Glu Leu Phe Gln
            115   120   125

Ala Ala Phe Leu Val Ala Asp Asp Ile Met Asp Gln Ser Leu Thr Arg
        130   135   140

Arg Gly Gln Leu Cys Trp Tyr Lys Lys Glu Gly Val Gly Leu Asp Ala
145   150   155   160

Ile Asn Asp Ser Phe Leu Leu Glu Ser Ser Val Tyr Arg Val Leu Lys
                165   170   175

Lys Tyr Cys Arg Gln Arg Pro Tyr Tyr Val His Leu Leu Glu Leu Phe
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Leu Gln Thr Ala Tyr Gln Thr Glu Leu Gly Gln Met Leu Asp Leu Ile
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Thr Ala Pro Val Ser Lys Val Asp Leu Ser His Phe Ser Glu Glu Arg
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Tyr Lys Ala Ile Val Lys Tyr Lys Thr Ala Phe Tyr Ser Phe Tyr Leu
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            275                 280                 285

Lys Val Gly Thr Asp Ile Gln Asp Asn Lys Cys Ser Trp Leu Val Val
            290                 295                 300

Gln Cys Leu Gln Arg Val Thr Pro Glu Gln Arg Gln Leu Leu Glu Asp
305                 310                 315                 320

Asn Tyr Gly Arg Lys Glu Pro Glu Lys Val Ala Lys Val Lys Glu Leu
            325                 330                 335

Tyr Glu Ala Val Gly Met Arg Ala Ala Phe Gln Gln Tyr Glu Glu Ser
            340                 345                 350

Ser Tyr Arg Arg Leu Gln Glu Leu Ile Glu Lys His Ser Asn Arg Leu
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            20                  25                  30


Ser His His Ser Tyr Tyr Gly Leu Cys Ser Thr Ser Cys Gln Val Tyr
        35                  40                  45


Asp Thr Ala Trp Val Ala Met Ile Pro Lys Thr Arg Asp Asn Val Lys
    50                  55                  60


Gln Trp Leu Phe Pro Glu Cys Phe His Tyr Leu Leu Lys Thr Gln Ala
65                  70                  75                  80
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Ala Asp Gly Ser Trp Gly Ser Leu Pro Thr Thr Gln Thr Ala Gly Ile
                85              90              95

Leu Asp Thr Ala Ser Ala Val Leu Ala Leu Leu Cys His Ala Gln Glu
            100             105             110

Pro Leu Gln Ile Leu Asp Val Ser Pro Asp Glu Met Gly Leu Arg Ile
            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
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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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                500                 505                 510

Arg Gly Phe Ser Trp Leu Lys Ser Cys Ser Phe His Ser Gln Asp Leu
        515                 520                 525

Thr Trp Thr Ser Lys Thr Ala Tyr Glu Val Gly Phe Val Ala Glu Ala
        530                 535                 540

Tyr Lys Leu Ala Ala Leu Gln Ser Ala Ser Leu Glu Val Pro Ala Ala
545                 550                 555                 560

Thr Ile Gly His Ser Val Thr Ser Ala Val Pro Ser Ser Asp Leu Glu
                565                 570                 575

Lys Tyr Met Arg Leu Val Arg Lys Thr Ala Leu Phe Ser Pro Leu Asp
                580                 585                 590

Glu Trp Gly Leu Met Ala Ser Ile Ile Glu Ser Ser Phe Phe Val Pro
                595                 600                 605

Leu Leu Gln Ala Gln Arg Val Glu Ile Tyr Pro Arg Asp Asn Ile Lys
        610                 615                 620

Val Asp Glu Asp Lys Tyr Leu Ser Ile Ile Pro Phe Thr Trp Val Gly
625                 630                 635                 640

Cys Asn Asn Arg Ser Arg Thr Phe Ala Ser Asn Arg Trp Leu Tyr Asp
                645                 650                 655

Met Met Tyr Leu Ser Leu Leu Gly Tyr Gln Thr Asp Glu Tyr Met Glu
                660                 665                 670

Ala Val Ala Gly Pro Val Phe Gly Asp Val Ser Leu Leu His Gln Thr
                675                 680                 685

Ile Asp Lys Val Ile Asp Asn Thr Met Gly Asn Leu Ala Arg Ala Asn
        690                 695                 700

Gly Thr Val His Ser Gly Asn Gly His Gln His Glu Ser Pro Asn Ile
705                 710                 715                 720

Gly Gln Val Glu Asp Thr Leu Thr Arg Phe Thr Asn Ser Val Leu Asn
                725                 730                 735

His Lys Asp Val Leu Asn Ser Ser Ser Ser Asp Gln Asp Thr Leu Arg
                740                 745                 750

Arg Glu Phe Arg Thr Phe Met His Ala His Ile Thr Gln Ile Glu Asp
        755                 760                 765

Asn Ser Arg Phe Ser Lys Gln Ala Ser Ser Asp Ala Phe Ser Ser Pro
770                 775                 780

Glu Gln Ser Tyr Phe Gln Trp Val Asn Ser Thr Gly Gly Ser His Val
785                 790                 795                 800

Ala Cys Ala Tyr Ser Phe Ala Phe Ser Asn Cys Leu Met Ser Ala Asn
                805                 810                 815

Leu Leu Gln Gly Lys Asp Ala Phe Pro Ser Gly Thr Gln Lys Tyr Leu
                820                 825                 830

Ile Ser Ser Val Met Arg His Ala Thr Asn Met Cys Arg Met Tyr Asn
        835                 840                 845

Asp Phe Gly Ser Ile Ala Arg Asp Asn Ala Glu Arg Asn Val Asn Ser
        850                 855                 860

Ile His Phe Pro Glu Phe Thr Leu Cys Asn Gly Thr Ser Gln Asn Leu
865                 870                 875                 880

Asp Glu Arg Lys Glu Arg Leu Leu Lys Ile Ala Thr Tyr Glu Gln Gly
                885                 890                 895

Tyr Leu Asp Arg Ala Leu Glu Ala Leu Glu Arg Gln Ser Arg Asp Asp
                900                 905                 910

Ala Gly Asp Arg Ala Gly Ser Lys Asp Met Arg Lys Leu Lys Ile Val
                915                 920                 925
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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

```
atggcggccg caccaggtaa aattgaaaat ggtactccaa aagatttgaa aactggtaat      60

gatttcgttt ctgctgctaa atctttgttg gatagagctt ttaaatctca tcattcttac     120

tacggtttgt gttctacttc ttgtcaagtt tacgatactg cttgggttgc tatgattcca     180

aagactagag ataacgttaa gcaatggttg ttcccagaat gtttccatta tttgttgaaa     240

actcaagctg ctgatggttc ttggggttct ttgccaacta ctcaaactgc tggtattttg     300

gatactgctt ctgctgtttt ggctttgttg tgtcatgctc aagaaccatt gcaaattttg     360

gatgtttctc agatgaaat gggtttgaga attgaacatg gtgttacttc tttgaaaaga     420

caattggctg tttggaatga tgttgaagat actaatcata ttggtgttga gttcatcatt     480

ccagctttgt tgtctatgtt ggaaaaagaa ttggatgttc atcttttga atttccatgt     540

agatctattt tggaaagaat gcatggtgaa aaattgggtc attttgattt ggaacaagtt     600

tatggtaaac atcttctttt gttgcattct ttggaagcat ccttggtaa attggatttt     660

gatagattgt ctcatcattt gtatcatggt tctatgatgg cttctccatc ttctactgct     720

gcttatttga ttggtgctac taaatgggat gatgaagctg aagattattt gagacatgtt     780

atgagaaatg gtgctggtca tggtaatggt ggtatttctg gtacttttcc aactactcat     840

ttcgaatgtt cttggatcat cgctactttg ttgaaggttg gttttacttt gaagcaaatt     900

gatggagatg gtttgagagg tttgtctact attttgttgg aagctcttag agatgaaaat     960

ggtgttattg gttttgctcc aagaactgct gatgttgatg atactgctaa ggctttgttg    1020

gctttgtctt tggttaacca accagtttct ccagatatca tgatcaaggt tttcgagggt    1080

aaagatcatt tcactacttt cggttctgaa agagatccat ctttgacttc taatttgcat    1140

gttttgttgt ctttgttgaa gcaatctaat ttgtctcaat accatccaca aatcttgaag    1200

actactttgt ttacttgtag atggtggtgg ggttctgatc attgtgttaa ggataagtgg    1260

aatttgtctc atttgtaccc aactatgttg ttggttgaag cattcactga agttttgcat    1320

ttgatcgatg gtggtgaatt gtcttctttg ttcgatgaat cttttaaatg caagatcggt    1380

ttgtctatct tccaagctgt ttttgagaatc atcttgactc aagataacga tggttcttgg    1440

agaggttata gagaacaaac ttgttatgct atttttggctt tggttcaagc tagacatgtt    1500

tgtttcttta ctcatatggt tgatagattg caatcttgtg ttgatagagg tttttcttgg    1560

ttgaaatctt gttcttttca ttctcaagat ttgacttgga cttctaaaac tgcttatgaa    1620

gttggttttg ttgctgaagc atacaaattg gctgctttgc aatctgcttc tttggaagtt    1680

ccagctgcta ctattggtca ttctgttact tctgctgttc atcttctga tttggaaaag    1740

tacatgagat tggttagaaa gactgctttg ttttctccat ggatgaatg gggtttgatg    1800

gcttctatta ttgaatcttc attttttcgtt ccattgttgc aagctcaaag agttgaaatc    1860
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tacccaagag ataacatcaa ggttgatgag gataagtatt tgtctatcat tccattcact      1920

tgggttggtt gtaacaacag atctagaact ttcgcttcta acagatggtt gtacgatatg      1980

atgtatttgt ctttgttggg ttaccaaact gatgaatata tggaagctgt tgctggtcca      2040

gttttcggag atgtttcttt gttgcatcaa actatcgata aagttattga taacactatg      2100

ggtaatttgg ctagagctaa cggtactgtt cattctggta atggtcatca acatgaatct      2160

ccaaacatcg gtcaagttga agatactttg actagattca ctaactctgt tttgaaccat      2220

aaggatgttt tgaactcttc ctcaagtgat caagatactt tgagaagaga gttcagaact      2280

tttatgcatg ctcatatcac tcaaatcgaa gataattcta gattttctaa gcaagctagt      2340

tctgatgctt tttcttctcc agaacaatct tattttcaat gggttaattc tactggtggt      2400

tctcatgttg cttgtgctta ttcttttgct ttttctaatt gtttgatgtc tgctaatttg      2460

ttgcagggta aagatgcttt cccatctggt actcaaaagt atttgatttc ttctgttatg      2520

agacatgcta ctaacatgtg tagaatgtac aacgatttcg gttctatcgc tagagataac      2580

gctgaaagaa acgttaattc tatccatttc ccagagttca ctttgtgtaa cggtacttct      2640

caaaatttgg atgaaagaaa ggaaagattg ttgaagatcg ctacttacga acaaggttat      2700

ttggatagag ctttggaggc acttgaaaga caatctagag atgatgctgg agatagagct      2760

ggttctaagg atatgagaaa attgaagatc gttaaattgt tttgtgatgt tactgatttg      2820

tatgatcaat gtatgttat  taaagatttg tcttcaagta tgaaataa                  2868
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<210> SEQ ID NO 10
<211> LENGTH: 2115
<212> TYPE: DNA
<213> ORGANISM: Artemisia annua

<400> SEQUENCE: 10

atgcaatcaa caacttccgt taagttatct cccttcgatc taatgacggc gttacttaac       60

ggcaaggtat ccttcgacac atcaaacaca tccgatacga atattccgtt agccgtgttt      120

atggagaatc gtgagctttt gatgattta actacttcgg ttgcggttct 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 ttgatttgga tgattatgcc      420

gctgaagatg atgagtatga ggagaagtta aagaaagaat ctcttgcttt tttctttta      480

gctacgtatg gagatggtga gccgacagat aatgctgcta gattctataa atggtttacc      540

gagggtgaag agaaaggtga atggcttgac aagcttcaat acgcagtgtt tggacttggt      600

aacagacagt atgagcattt caacaagatt gctaaggtgg tcgatgaaaa acttgtggaa      660

cagggtgcaa agcgccttgt tcctgttggc atgggagacg atgatcaatg tatcgaagac      720

gacttcactg catggaaaga gttggtgtgg cctgagttgg atcaattact tcgtgatgag      780

gatgatacat ctgttgccac tccatacaca gctgctgttg cagaataccg tgttgtgttc      840

catgataaac cagagacata tgatcaggat caactgacaa atggccatgc tgttcatgat      900

gctcaacatc catgcagatc caatgtcgct gtcaaaaagg agctccattc ccctctatct      960

gaccggtctt gcactcattt ggaatttgat atctctaata ctggattatc gtatgaaact     1020

ggggaccatg ttggagtcta tgttgagaat ctaagtgaag ttgtggacga agctgaaaaa     1080

ttaataggtt taccgccgca cacttatttc tcagtacaca ctgataacga agacgggaca     1140
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ccacttggtg gagcatcttt gccacctcct ttccctccat gcactttaag aaaagcattg     1200

gcttcctatg ctgatgtttt gagctctcct aaaaagtcag ctttgcttgc tttagctgct     1260

catgccactg attctactga agctgataga ctgaaatttc ttgcgtctcc tgctggaaag     1320

gatgaatatg ctcagtggat agttgcaagc cacagaagtc tccttgaggt catggaggcc     1380

ttcccatcag ctaagcctcc gcttggtgtt tttttttgcat ctgttgcccc acgtttgcag     1440

ccgagatact attccatttc ttcttcccca agtttgcgc caaataggat tcatgtaact     1500

tgtgcattag tgtatgagca aacaccgtca ggccgcgttc acaagggagt ctgttcaaca     1560

tggatgaaga atgccgtgcc tatgacagaa agccaggatt gcagttgggc cccaatttat     1620

gttagaacat ccaatttcag acttccttct gatcctaagg tcccagttat catgattggc     1680

ccaggcactg gattggctcc atttagaggt ttccttcagg aaaggttagc tcagaaggaa     1740

gctgggactg agctcggaac agccatttta ttcttcggat gcaggaatcg caaagtggat     1800

ttcatatatg aagacgagct taataatttt gtggagacgg gggctctttc cgagcttgtt     1860

acggccttct ctcgtgaagg tgccactaag gagtacgtgc aacacaagat gactcagaag     1920

gcttcggata tctggaattt tctctctgag ggagcatatt tgtatgtttg cggtgatgcc     1980

aaaggcatgg ccaaagatgt acatcggact ctgcacacaa ttgtgcaaga acagggatct     2040

ctagactcct caaaggcgga gctctacgtg aagaatctac aaatggcagg aagatatctc     2100

cgtgatgtat ggtaa                                                      2115
```

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

<400> SEQUENCE: 11

```
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
```

```
        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
```

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

<400> SEQUENCE: 12

atggccttct tctccatgat ctccattctc cttggctttg ttatctcctc cttcatcttc        60

atcttcttct tcaagaaact tctctccttc tccagaaaga acatgtctga agtctccact       120

ctcccctctg ttccagtggt accagggttt cctgttattg ggaacttgct gcaactaaaa       180

gagaagaaac ctcacaagac tttcactaga tggtcagaga tttatggtcc tatttactct       240

ataaagatgg gttcttcttc tcttattgtc ctcaattcta ctgagactgc caaagaggcc       300

atggtgacgc ggttttcgtc tatctcaacg aggaagttgt caaatgcgtt gacagtcctt       360

acttgtgaca atctatggt tgctactagt gattatgatg atttccacaa gttggtgaaa        420

cggtgtctct tgaacggtct ttgggtgct aatgcacaga acgaaaaag acattacaga         480

gatgcactca ttgaaaatgt gtcttccaag ttgcatgccc atgctaggga ccatccacaa       540

gaacctgtaa 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 agcgaaaaca       900

ctaaccaagg agcaaattgc tatcttggtt tgggagacga ttatcgagac agctgacact       960

actttggtta caactgaatg ggccatctat gagctcgcta agcatccaag tgtccaagat      1020

cgtctgtgca agaaatccaa aaatgtctgc ggaggagaaa agttcaaaga gagcaattg       1080

tctcaagttc cttatctcaa tggagtcttt catgaaacgc ttaggaaata cagtcctgct      1140

cctctagttc ccattcgcta cgcccacgag gatacgcaaa tcggaggcta tcatgtccct      1200

gcaggaagtg agattgcaat aaacatatat ggatgcaaca tggataagaa gcgttgggag      1260

agaccagagg actggtggcc ggagcggttt cttgatgatg caaatatga aacgtcagat       1320

cttcacaaga caatggcgtt tggagcggga aagagggttt gtgctggtgc tcttcaagca      1380

tctctcatgg caggcattgc tattggaaga ttagtgcaag aattcgagtg aagcttaga       1440

gatggcgaag aagagaatgt ggatacatat ggcttgacct ctcagaagct ttatcctctt      1500

atggctatta tcaatccaag gcgttcttaa                                        1530
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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

Met Ala Phe Phe Ser Met Ile Ser Ile Leu Leu Gly Phe Val Ile Ser
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
```

```
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              480

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

<400> SEQUENCE: 14

atgagtaagt ccaacagcat gaacagtacc agccatgaaa cgttattcca gcagctcgtc      60

ttaggtcttg acagaatgcc gctaatggac gttcactggc tgatctacgt ggcctttggc     120

gcttggttat gctcttatgt catccatgtc ctatcgtcct cttctacagt caaagtgccc     180

gtcgtaggct accgcagcgt ctttgagcct acatggcttc tccgtttgcg ctttgtttgg     240

gaaggggat ctatcatcgg ccaaggctac aacaaattta aagactctat cttccaggtg      300

cgaaagcttg gtaccgatat cgtcatcatc ccgccaaact acatcgatga ggtcagaaag     360

ctgtcccaag acaagactcg ctcggtcgag cccttcatca atgactttgc gggacagtat     420

acacggggca tggtctttct gcaaagtgat ttgcagaacc gtgtgattca gcagcggttg     480

acgccaaaac tcgtatcgtt gacaaaggta atgaaggagg agcttgacta tgccttgacc     540

aaagagatgc ctgacatgaa gaatgatgaa tgggttgaag tcgacatttc ttccatcatg     600

gtcaggctca tatcacgcat ctcagccaga gtgtttctcg tccagagca ctgccgcaac      660

caagaatggt tgacgaccac tgcagagtac agcgagagcc tgttcataac tggctttatt     720

ctccgcgttg tccccatat tctaagacca ttcatagccc cgctgctacc tcctacaga      780

acactacttc gcaacgtctc gtcaggtcga gagttattg gagacatcat tcgctcccag      840

caaggtgatg caacgagga catcctgtca tggatgaggg atgctgcgac aggggaagaa      900

aagcaaattg acaacattgc ccagcggatg cttatcctga gtctcgcgtc tattcacact     960

acggcaatga cgatgacgca tgctatgtat gacttatgtg cttgccctga gtacatagag    1020

cctcttagag atgaggtcaa aagtgtcgtt ggcgctagtg gttgggacaa gacggcgttg    1080

aatcgattcc acaaactcga cagctttctc aaagagtcac aacgcttcaa ccccgtgttc    1140

ctcttaacgt tcaatcgcat ttatcaccaa tccatgacac tctcagatgg caccaacatc    1200

ccatcaggca ctcgcatcgc ggttccctct cacgcgatgc ttcaggactc agcgcatgtc    1260

ccaggcccga cgccaccaac cgagtttgat ggatttagat actcaaagat tcgctcagac    1320

tcaaactatg cacagaaata tctcttctcc atgactgatt ctagtaacat ggcgtttggg    1380

tatgggaaat acgcctgccc agggcggttc tatgcatcta atgagatgaa gctgactttg    1440
```

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```
gcgatactcc ttttacaatt tgagttcaag ttgccagatg ggaaaggaag 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 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 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 gtccattggt taatctatgt tgcctttggt     120

gcttggttat gttcttacgt aatacacgtc ttgtcttcat ccagtacagt taaagtacca     180

gttgtaggtt atagatcagt tttcgaacct acctggttgt taagattgag atttgtttgg     240

gaaggtggtt ccatcatcgg tcaaggttac aacaaattca aggatagtat cttccaagtt     300

agaaagttag gtacagacat agtaatcatt ccacctaact acatcgatga agttagaaaa     360

ttgtctcaag acaagactag atcagtagaa ccttttatta cgatttcgc aggtcaatac     420

acaagaggta tggtcttttt gcaatccgac ttacaaaaca gagttattca acaaagattg     480

accccaaaat tggtttcttt aactaaagta atgaaggaag aattggatta cgccttaact     540

aaagaaatgc tgatatgaa gaacgacgaa tgggtcgaag ttgatatttc ttctatcatg     600

gttagattaa tatccagaat cagtgctaga gtcttcttgg gtcctgaaca ttgcagaaat     660

caagaatggt tgactacaac cgcagaatat tccgaaagtt tgtttatcac aggtttcatt     720

ttgagagtcg ttccacatat cttgagacct tttatcgcac cattgttgcc ttcatacaga     780

actttgttga aaacgtatc cagtggtaga agagtcatcg tgacattat 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 ctccttttta aaagaaagtc aaagattcaa tccagttttc      1140

ttgttgacct ttaacagaat ctatcaccaa tccatgactt taagtgatgg tacaaatatc      1200

ccatctggta ctagaattgc agttccttcc catgccatgt tgcaagatag tgcccacgtt      1260

ccaggtccta caccacctac cgaatttgat ggtttcagat actctaagat cagatctgac      1320

tcaaactacg ctcaaaagta cttattctca atgactgatt cttcaaacat ggcttttggt      1380

tatggtaaat acgcatgtcc aggtagattt tacgcctcta acgaaatgaa gttgacattg      1440

gctatcttgt tgttgcaatt cgagtttaaa ttgccagatg gtaaaggtag acctagaaat      1500

attaccatag attctgacat gatacctgac ccaagagcaa gattatgcgt tagaaaaaga      1560

agtttga                                                                 1567
```

<210> SEQ ID NO 17
<211> LENGTH: 2607
<212> TYPE: DNA
<213> ORGANISM: Abies grandis

<400> SEQUENCE: 17

```
atggccatgc cttcctcttc attgtcatca cagattccca ctgctgctca tcatctaact        60

gctaacgcac aatccattcc gcatttctcc acgacgctga atgctggaag cagtgctagc       120

aaacggagaa gcttgtacct acgatggggt aaaggttcaa acaagatcat tgcctgtgtt       180

ggagaaggtg gtgcaacctc tgttccttat cagtctgctg aaaagaatga ttcgctttct       240

tcttctacat tggtgaaacg agaatttcct ccaggatttt ggaaggatga tcttatcgat       300

tctctaacgt catctcacaa ggttgcagca tcagacgaga agcgtatcga gacattaata       360

tccgagatta agaatatgtt tagatgtatg ggctatggcg aaacgaatcc ctctgcatat       420

gacactgctt gggtagcaag gattccagca gttgatggct ctgacaaccc tcactttcct       480

gagacggttg aatggattct tcaaaatcag ttgaaagatg ggtcttgggg tgaaggattc       540

tacttcttgg catatgacag aatactggct acacttgcat gtattattac ccttaccctc       600

tggcgtactg gggagacaca agtacagaaa ggtattgaat tcttcaggac acaagctgga       660

aagatggaag atgaagctga tagtcatagg ccaagtggat ttgaaatagt atttcctgca       720

atgctaaagg aagctaaaat cttaggcttg gatctgcctt acgatttgcc attcctgaaa       780

caaatcatcg aaaagcggga ggctaagctt aaaaggattc ccactgatgt tctctatgcc       840

cttccaacaa cgttattgta ttctttggaa ggtttacaag aaatagtaga ctggcagaaa       900

ataatgaaac ttcaatccaa ggatggatca tttctcagct ctccggcatc tacagcggct       960

gtattcatgc gtacagggaa caaaaagtgc ttggatttct tgaactttgt cttgaagaaa      1020

ttcggaaacc atgtgccttg tcactatccg cttgatctat ttgaacgttt gtgggcggtt      1080

gatacagttg agcggctagg tatcgatcgt catttcaaag aggagatcaa ggaagcattg      1140

gattatgttt acagccattg ggacgaaaga ggcattggat gggcgagaga gaatcctgtt      1200

cctgatattg atgatacagc catgggcctt cgaatcttga gattacatgg atacaatgta      1260

tcctcagatg tttttaaaaac atttagagat gagaatgggg agttcttttg cttcttgggt      1320

caaacacaga gaggagttac agacatgtta aacgtcaatc gttgttcaca tgtttcattt      1380

ccgggagaaa cgatcatgga agaagcaaaa ctctgtaccg aaaggtatct gaggaatgct      1440

ctggaaaatg tggatgcctt tgacaaatgg gctttttaaaa agaatattcg gggagaggta      1500

gagtatgcac tcaaatatcc ctggcataag agtatgccaa ggttggaggc tagaagctat      1560
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```
attgaaaact atgggccaga tgatgtgtgg cttggaaaaa ctgtatatat gatgccatac    1620

atttcgaatg aaaagtattt agaactagcg aaactggact tcaataaggt gcagtctata    1680

caccaaacag agcttcaaga tcttcgaagg tggtggaaat catccggttt cacggatctg    1740

aatttcactc gtgagcgtgt gacgaaaata tatttctcac cggcatcctt tatctttgag    1800

cccgagtttt ctaagtgcag agaggtttat acaaaaactt ccaatttcac tgttatttta    1860

gatgatcttt atgacgccca tggatcttta gacgatctta agttgttcac agaatcagtc    1920

aaaagatggg atctatcact agtggaccaa atgccacaac aaatgaaaat atgttttgtg    1980

ggtttctaca atacttttaa tgatatagca aaagaaggac gtgagaggca agggcgcgat    2040

gtgctaggct acattcaaaa tgtttggaaa gtccaacttg aagcttacac gaaagaagca    2100

gaatggtctg aagctaaata tgtgccatcc ttcaatgaat acatagagaa tgcgagtgtg    2160

tcaatagcat tgggaacagt cgttctcatt agtgctcttt tcactgggga ggttcttaca    2220

gatgaagtac tctccaaaat tgatcgcgaa tctagatttc ttcaactcat gggcttaaca    2280

gggcgtttgg tgaatgacac caaaacttat caggcagaga gaggtcaagg tgaggtggct    2340

tctgccatac aatgttatat gaaggaccat cctaaaatct ctgaagaaga agctctacaa    2400

catgtctata gtgtcatgga aaatgccctc gaagagttga atagggagtt tgtgaataac    2460

aaaataccgg atatttacaa 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

<400> SEQUENCE: 18

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
```

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<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

atggctatgc ctagttcttc tctcagttca caaattccaa ctgctgctca ccacttaaca        60

gcaaacgcac aaagtattcc acattttct actacactta atgctggatc tagtgcttct       120

aagaggagat cattgtattt gagatgggga aaaggatcca acaagattat tgcatgcgtg       180

ggagaaggag gtgcaacatc agttccttac caatctgctg agaagaatga ttctttaagt       240

tcttcaacac ttgtgaaaag ggagtttcca cctggttttt ggaaagatga tctcattgat       300

tctttaactt cttcccataa agtggctgca tccgatgaaa aaaggattga gactctcatt       360

tctgaaatta agaacatgtt tagatgtatg ggttacggag aaactaaccc ttctgcttac       420

gatacagctt gggttgctag gattccagct gtggatggta gtgataaccc acatttttcct       480

gagactgttg aatggattct tcagaatcag ctcaaagatg gttcttgggg agaaggattc       540
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tatttcttag cttacgatag aattttggca actttggctt gcattattac tttgacactt      600

tggagaactg gtgaaacaca agttcagaag ggtattgaat ttttcaggac tcaagcagga      660

aagatggagg atgaggctga tagtcacaga ccttcaggtt tcgagattgt gtttccagca      720

atgttgaaag aggctaagat tcttggattg gatcttcctt acgatttgcc atttctcaag      780

caaattattg agaaaagaga agctaagctc aaaaggattc ctacagatgt tctctacgca      840

ttaccaacaa ctcttttgta ttctttggaa ggacttcaag aaattgttga ttggcaaaag      900

attatgaaac tccaaagtaa ggatggatct tttctctcat ctcctgcttc tactgctgct      960

gtttttatga ggacaggtaa caagaagtgt ttagatttct taaatttcgt gctcaaaaag     1020

tttggaaatc atgttccatg ccactatcct cttgatttat ttgaaagact ttgggctgtt     1080

gatacagtgg agaggcttgg tattgatagg cattttaaag aagaaattaa ggaggcattg     1140

gattatgttt actctcattg ggatgagaga ggaattggat gggctagaga aaaccctgtt     1200

cctgatattg atgatacagc aatgggtctt agaattttaa gattgcatgg atacaatgtt     1260

tcttcagatg tttttaaaaac atttagagat gagaatggag agttcttctg ctttttaggt     1320

cagacacaaa ggggagttac agatatgttg aatgttaaca gatgttctca tgttagtttc     1380

cctggtgaga ctattatgga ggaagctaag ttgtgcacag agagatatct tagaaatgca     1440

ttggagaatg ttgatgcttt cgataaatgg gcattcaaaa agaatattag gggtgaagtg     1500

gaatatgctc tcaagtaccc atggcataag tctatgccta ggttggaggc tagatcatat     1560

attgagaact atggtcctga tgatgtttgg cttggaaaaa cagtgtacat gatgccttat     1620

atttcaaatg aaaaatacct tgaactcgct aagctcgatt ttaataaggt tcagtctatt     1680

caccaaactg agttgcagga tttaaggagg tggtggaaat cttcaggatt cactgatctt     1740

aattttacta gagagagagt tactgagatt tacttctcac ctgctagttt tattttcgaa     1800

ccagagttct caaaatgtag agaggtttat acaaaaacta gtaatttcac agttattttg     1860

gatgatttgt acgatgctca cggtagtctc gatgatctta aacttttttac agaatcagtt     1920

aaaagatggg atttgtcatt agttgatcaa atgccacaac aaatgaagat ttgtttttgtg     1980

ggattttaca atacttttaa tgatattgct aaagagggta gggagagaca aggtagagat     2040

gttcttggat atattcagaa cgtttggaaa gtgcagttag aggcttatac aaaagaagca     2100

gagtggtctg aggcaaagta tgtgccatct ttcaatgagt acattgaaaa cgcatctgtg     2160

agtattgctc tcggtactgt tgtgcttatt tcagctttat ttacaggaga ggtgcttact     2220

gatgaagtgt tgtccaaaat tgatagggag agtagatttc ttcaacttat gggtcttaca     2280

ggtaggcttg ttaatgatac aaagacttat caagctgaaa ggggtcaagg agaagttgct     2340

tctgctattc aatgttatat gaaggatcat cctaaaattt ctgaagaaga agcattgcaa     2400

catgtttatt cagtgatgga aaacgcactc gaagaattaa atagggagtt cgttaacaac     2460

aaaattccag atatttataa aagacttgtt tttgaaactg ctagaattat gcagctcttt     2520

tacatgcaag gtgatggatt aactttgtcc catgatatgg aaattaagga gcacgttaaa     2580

aattgtttgt tccaacctgt tgcataa                                         2607
```

<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

gagctcagtt tatcattatc aatactcgcc atttcaaaga atacgtaaat aattaatagt      60

agtgattttc ctaactttat ttagtcaaaa aattagcctt ttaattctgc tgtaacccgt     120

acatgcccaa aataggggc gggttacaca gaatatataa catcgtaggt gtctgggtga      180

acagtttatt cctggcatcc actaaatata atggagcccg cttttttaagc tggcatccag    240

aaaaaaaaag aatcccagca ccaaaatatt gttttcttca ccaaccatca gttcataggt     300

ccattctctt agcgcaacta cagagaacag gggcacaaac aggcaaaaaa cgggcacaac     360

ctcaatggag tgatgcaacc tgcctggagt aaatgatgac acaaggcaat tgacccacgc     420

atgtatctat ctcattttct tacaccttct attaccttct gctctctctg atttggaaaa     480

agctgaaaaa aaaggttgaa accagttccc tgaaattatt cccctacttg actaataagt     540

atataaagac ggtaggtatt gattgtaatt ctgtaaatct atttcttaaa cttcttaaat     600

tctacttta tagttagtct ttttttttagt tttaaaacac cagaacttag tttcgacg       658


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

<400> SEQUENCE: 21

ttctggcaac caaacccata tacatcggga ttcctataat accttcgttg gtctccctaa      60

catgtaggtg gcggaggga gatatacaat agaacagata ccagacaaga cataatgggc       120

taaacaagac tacaccaatt acactgcctc attgatggtg gtacataacg aactaatact      180

gtagccctag acttgatagc catcatcata tcgaagtttc actacccttt ttccatttgc      240

catctattga agtaataata ggcgcatgca acttctttc tttttttttc ttttctctct       300

cccccgttgt tgtctcacca tatccgcaat gacaaaaaaa tgatggaaga cactaaagga     360

aaaaattaac gacaaagaca gcaccaacag atgtcgttgt tccagagctg atgaggggta     420

tctcgaagca cacgaaactt tttccttcct tcattcacgc acactactct ctaatgagca     480

acggtatacg gccttccttc cagttacttg aatttgaaat aaaaaaaagt ttgctgtctt     540

gctatcaagt ataaatagac ctgcaattat taatctttg tttcctcgtc attgttctcg      600

ttccctttct tccttgtttc tttttctgca caatatttca agctatacca agcatacaat     660

caacta                                                                 666


<210> SEQ ID NO 22
<211> LENGTH: 408
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: engineered sequence

<400> SEQUENCE: 22

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cgcgcatcgc cgtaccactt caaaacaccc aagcacagca tactaaattt cccctctttc      120

ttcctctagg gtgtcgttaa ttacccgtac taaaggtttg gaaaagaaaa aagagaccgc     180

ctcgtttctt tttcttcgtc gaaaaaggca ataaaaattt ttatcacgtt tcttttttctt    240

gaaaatttt tttttgattt ttttctcttt cgatgacctc ccattgatat ttaagttaat      300

aaacggtctt caatttctca agtttcagtt tcattttct tgttctatta caacttttt       360
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tacttcttgc tcattagaaa gaaagcatag caatctaatc taagtttt              408


<210> SEQ ID NO 23
<211> LENGTH: 481
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: engineered sequence

<400> SEQUENCE: 23

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tctgtattta aaacactttt gtattatttt tcctcatata tgtgtatagg tttatacgga     120

tgatttaatt attacttcac caccctttat ttcaggctga tatcttagcc ttgttactag     180

ttagaaaaag acatttttgc tgtcagtcac tgtcaagaga ttcttttgct ggcatttctt     240

ctagaagcaa aaagagcgat gcgtcttttc cgctgaaccg ttccagcaaa aaagactacc     300

aacgcaatat ggattgtcag aatcatataa aagagaagca aataactcct tgtcttgtat     360

caattgcatt ataatatctt cttgttagtg caatatcata tagaagtcat cgaaatagat     420

attaagaaaa acaaactgta caatcaatca atcaatcatc acataaatcc ggacgacaga     480

g                                                                     481


<210> SEQ ID NO 24
<211> LENGTH: 421
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: engineered sequence

<400> SEQUENCE: 24

ggatcctcaa aacccttaaa aacatatgcc tcaccctaac atattttcca attaaccctc      60

aatatttctc tgtcacccgg cctctatttt ccattttctt ctttacccgc cacgcgtttt     120

tttctttcaa attttttttct tccttcttct ttttcttcca cgtcctcttg cataaataaa     180

taaaccgttt tgaaaccaaa ctcgcctctc tctctccttt ttgaaatatt tttgggtttg     240

tttgatcctt tccttcccaa tctctcttgt ttaatatata ttcatttata tcacgctctc     300

tttttatctt cctttttttc ctctctcttg tattcttcct tccccttct actcaaacca      360

agaagaaaaa gaaaaggtca atctttgtta aagaatagga tcttctacta catcagcttt     420

t                                                                     421
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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 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:

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.

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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