Cytochrome P450s and Uses Thereof

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(54) CYTOCHROME P450S AND USES THEREOF

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None

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(57) ABSTRACT

The invention features isolated cytochrome P450 polypeptides and nucleic acid molecules, as well as expression vectors and transgenic plants containing these molecules. In addition, the invention features uses of such molecules in methods of increasing the level of resistance against a disease caused by a plant pathogen in a transgenic plant, in methods for producing altered compounds, for example, hydroxylated compounds, and in methods of producing isoprenoid compounds.

16 Claims, 11 Drawing Sheets
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FIG. 1

farnesyl diphosphate → 5-epi-aristolochene

1-deoxycapsidiol (5-hydroxy-5-epi-aristolochene)

3-deoxycapsidiol (1-hydroxy-5-epi-aristolochene)

capsidiol

+EAS

+ NADPH + O₂
FIG. 2

Enzyme activity (% of maximum) vs. Time after elicitation (h)

- 0
- 25
- 50
- 75
- 100
FIG. 4A

KETLRLX, PFGXGRXCP(A/G)

N-term

C-term

FXPERF

FIG. 4B

KETLRLH-for 5'-AARGARACIYTIMGIYTIACA-3'
KETLRLY-for 5'-AARGARACIYTIMGIYTIATA-3'
KETLRLR-for 5'-AARGARACIYTIMGIYTIMG-3'
FXPERF-for 5'-TTYIIICCGIARMGITY-3'
FXPERF-rev 5'-RAAIKYTCIGIIIIIRA-3'
GRRXCP(A/G)-for 5'-GGIMGIIIITGYCCIGS-3'
PFGXGRR-rev 5'-CKICKICCIICCCRAAIGG-3'
T7 5'-GTAAATAGACTCATAAGG-3'
T3 5'-CAATTAACCCTCATAAGG-3'

FIG. 4C

600
500
400
300
CYTOCHROME P450s AND USES THEREOF

RELATED APPLICATIONS

This application is a continuation of U.S. patent application Ser. No. 12/182,000, filed Jul. 29, 2008, now U.S. Pat. No. 8,263,362 which is a continuation of U.S. patent application Ser. No. 10/897,559, filed Mar. 8, 2002 (now issued U.S. Pat. No. 7,405,857), which claims the benefit of U.S. Provisional Application Nos. 60/274,421 and 60/275,597, filed on Mar. 9, 2001 and Mar. 13, 2001, respectively, all of which are hereby incorporated by reference.

FIELD OF THE INVENTION

This invention relates to cytochrome P450s and uses thereof.

BACKGROUND OF THE INVENTION

Cytochrome P450s encompass a superfamily of oxidases responsible for the oxidation of numerous endobiotics and thousands of xenobiotics. In addition, plants, cytochrome P450s play important roles in wounding healing, pest resistance, signaling, and anti-microbial and anti-fungal activity.

Capsidil is a bicyclic, dihydroxyxylated sesquiterpene produced by many Solanaceae species in response to a variety of environmental stimuli, including exposure to UV (Baek et al., Plant Cell Physiol. 389:899-904, 1998) and infection by microorganisms (Molot et al., Physiol. Plant Pathol. 379:389, 1981; Stolle et al., Phytopathology 78:1193-1197, 1988; Keller et al., Planta. 205:467-476, 1998). It is the primary antibiotic or phytoalexin produced in tobacco in response to fungal elicitation and is derived from the isoprenoid pathway via its hydrocarbon precursor, 5-epi-aristolochene (FIG. 1). Several of the biosynthetic enzymes leading up to 5-epi-aristolochene formation have been studied (Chappell, Annu. Rev. Plant Physiol. Plant Mol. Biol. 46:521-547, 1995), especially 5-epi-aristolochene synthase (BAS) (Vogeli and Chappell, Plant Physiol. 88:1291-1296, 1988; Baek and Chappell, Proc. Natl. Acad. Sci. U.S.A. 93:6841-6845, 1996; Mathis et al., Biochemistry 36:8340-8348, 1997; Stark et al., Science 277:1815-1820, 1997). BAS commits carbon to sesquiterpene metabolism by catalyzing the cyclization of farnesyl diphosphate (FPP) to 5-epi-aristolochene. However, until the present invention, the enzyme(s) responsible for the conversion of 5-epi-aristolochene to capsidil has yet to be fully identified and characterized.

Biochemical evidence from previous studies in tobacco (Whitehead et al., Phytochemistry 28:775-779, 1989) and green pepper (Hoshino et al., Phytochemistry 38:609-613, 1995) have suggested that the oxidation of 5-epi-aristolochene to capsidil occurs in a two step process with one of the hydroxylation steps being constitutive and the other being mediated by an elicitor-inducible cytochrome P450 (FIG. 1). Because 1-deoxycapsidil had been isolated from natural sources (Watson et al., Biochem. Soc. Trans. 11:589, 1983), Whitehead et al. (Phytochemistry 28:775-779, 1989), surmised that perhaps the biosynthesis of this intermediate was due to pathogen induction of a corresponding hydroxylase. They therefore prepared synthetic 1-deoxycapsidil and reported a modest conversion of this compound to capsidil when fed to control or unelicited tobacco cell cultures. This was further supported by their observation that radiolabeled 5-epi-aristolochene was only converted to capsidil when fed to elicitor-induced cell cultures but not control cultures. Whitehead et al. (Phytochemistry 28:775-779, 1989) therefore concluded that the 3-hydroxylase, responsible for hydroxylation of 5-epi-aristolochene at C3 to generate 1-deoxycapsidil, was pathogen/elicitor inducible, while the 1-hydroxylase, responsible for hydroxylating 1-deoxycapsidil at the C1 to generate capsidil, was constitutive. Hoshino et al. (Phytochemistry 38:609-613, 1995) added to the observations of Whitehead et al. (Phytochemistry 28:775-779, 1989) by directly measuring 3-hydroxylase activity in microsomal preparations of arachidonic acid-elicited Capsicum annuum fruits and seedlings. These assays consisted of incubating 5-epi-aristolochene with microsomal preparations and subsequently determining the amount of 1-deoxycapsidil generated by a combination of thin-layer chromatography (TLC) separations and gas chromatography (GC). Their evidence demonstrated that the conversion of 5-epi-aristolochene to 1-deoxycapsidil was dependent on both NADPH and O₂; and that 1-deoxycapsidil accumulation in vitro was arrested by the P450 antagonists carbon monoxide (Omura and Sato, J. Biol. Chem. 239:2370-2378, 1964), ancamidol (Coolbaugh et al., Plant Physiol. 62:571-576, 1978), and ketaconazole (Rademacher, Annu. Rev. Plant Physiol. Plant Mol. Biol. 51:551-561, 2000).

Recent results suggest that the hydroxylation of 5-epi-aristolochene is an important regulated step in capsidil biosynthesis. In studies to evaluate the effectiveness of methyl jasmonate as an inducer of capsidil biosynthesis in tobacco cell cultures, Mandujano-Chavez et al. (Arch. Biochem. Biophys. 381:285-294, 2000), reported that the modest accumulation of this phytoalexin was accompanied by a strong induction of EAS. This result implied that steps before or after the sesquiterpenecyclase reaction were limiting. Using an in vivo assay measuring the conversion rate of radiolabeled 5-epi-aristolochene to capsidil, a very limited induction of the hydroxylase activity was observed in plants treated with methyl jasmonate relative to that in fungal elicitor-treated cells. This result pointed to the hydroxylase reactions as a potentially limiting step in capsidil biosynthesis.

SUMMARY OF THE INVENTION

In one aspect, the invention features several isolated cytochrome P450 polypeptides (such as CYP71D20, CYP71D21, CYP73A27, CYP73A28, and CYP92A5, and P450s having substantial identity to these polypeptides), as well as isolated nucleic acid molecules that encode these P450s.

In related aspects, the invention features a vector (such as an expression vector) including an isolated nucleic acid molecule of the invention and a cell (for example, a prokaryotic cell, such as Agrobacterium or E. coli, or a eukaryotic cell, such as a mammalian, insect, yeast, or plant cell) including the isolated nucleic acid molecule or vector.

In yet another aspect, the invention features a transgenic plant or transgenic plant component including a nucleic acid molecule of the invention, wherein the nucleic acid molecule is expressed in the transgenic plant or the transgenic plant component. Preferably, the transgenic plant or transgenic plant component is an angiosperm plant (for example, a monocot or dicot). In preferred embodiments, the transgenic plant or transgenic plant component is a solanaceous, maize, rice, or cruciferous plant or a component thereof. The invention further includes a seed produced by the transgenic plant or transgenic plant component, or progeny thereof.

In another aspect, the invention features a method of providing an increased level of resistance against a disease caused by a plant pathogen in a transgenic plant. The method involves: (a) producing a transgenic plant cell including the nucleic acid molecule of the invention integrated into the
genome of the transgenic plant cell and positioned for expression in the plant cell; and (b) growing a transgenic plant from the plant cell wherein the nucleic acid molecule is expressed in the transgenic plant and the transgenic plant is thereby provided with an increased level of resistance against a disease caused by a plant pathogen.

In another aspect, the invention features a method for producing an altered compound, the method including the steps of contacting the compound with one or more of the isolated polypeptides disclosed herein under conditions allowing for the hydroxylation, oxidation, demethylation, or methylation of the compound and recovering the altered compound.

In still another aspect, the invention features a hydroxylation agent including any of the isolated polypeptides disclosed herein.

In yet another embodiment, the invention features an isolated nucleic acid molecule that specifically hybridizes under highly stringent conditions to the complement of any one of the sequences described in SEQ ID NO:2 (CYP71D20), SEQ ID NO:4 (CYP71D21), SEQ ID NO:6 (CYP73A27), SEQ ID NO:8 (CYP73A28), or SEQ ID NO:12 (CYP92A5), wherein such a nucleic acid molecule encodes a cytochrome P450 polypeptide.

In another aspect, the invention features a host cell expressing a recombinant isoformid synthase and a recombinant cytochrome P450. In preferred embodiments, the host cell further expresses, independently or in combination, a recombinant acetyltransferase, methyltransferase, or fatty acyltransferase. In other preferred embodiments, the host expresses an endogenous or recombinant cytochrome reductase. Preferably, the host cell is a yeast cell, a bacterial cell, an insect cell, or a plant cell.

In a related aspect, the invention features a method for producing an isoformid compound, the method including the steps of: (a) culturing a cell that expresses a recombinant isoformid synthase and a recombinant cytochrome P450 under conditions wherein the isoformid synthase and the cytochrome P450 are expressed and catalyze the formation of an isoformid compound not normally produced by the cell; and (b) recovering the isoformid compound. In preferred embodiments, the host cell further expresses a recombinant acetyltransferase, a recombinant methyltransferase, or a recombinant fatty acyltransferase. In other preferred embodiments, the host cell expresses an endogenous or recombinant cytochrome reductase. Preferably, the host cell is a yeast cell, a bacterial cell, an insect cell, or a plant cell.

In yet another aspect, the invention features an isoformid compound produced according to the above-mentioned methods.

By “P450 polypeptide,” “cytochrome P450,” or “P450” is meant a polypeptide that contains a heme-binding domain and shows a CO absorption spectra peak at 450 nm according to standard methods, for example, those described herein. Such P450s may also include, without limitation, hydroxy-lase activity, dual hydroxy-lase activity, demethy-lase activity, or oxidase activity. Such enzymatic activities are determined using methods well known in the art.

By “polypeptide” is meant any chain of amino acids, regardless of length or post-translational modification (for example, glycosylation or phosphorylation).

By “substantially identical” is meant a polypeptide or nucleic acid exhibiting at least 80% or 85%, preferably 90%, more preferably 95%, and most preferably 97%, or even 98% identity to a reference amino acid sequence (for example, the amino acid sequence shown in SEQ ID NO:8.1, 3, 5, 7 and 11) or nucleic acid sequence (for example, the nucleic acid sequences shown in SEQ ID NO:8.4, 6, 8 and 12, respectively). For polypeptides, the length of comparison sequences will generally be at least 16 amino acids, preferably at least 20 amino acids, more preferably at least 25 amino acids, and most preferably 35 amino acids. For nucleic acids, the length of comparison sequences will generally be at least 50 nucleotides, preferably at least 60 nucleotides, more preferably at least 75 nucleotides, and most preferably 110 nucleotides.

Sequence identity is typically measured using sequence analysis software (for example, Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, Wis. 53705, BLAST, or PILEUP/PRETTYBOX programs). Such software matches identical or similar sequences by assigning degrees of homology to various substitutions, deletions, and/or other modifications. Conservative substitutions typically include substitutions within the following groups: glycine, alanine, valine, isoleucine, leucine, aspartic acid, glutamic acid, asparagine, glutamine; serine, threonine, lysine, arginine, and phenylalanine, tyrosine.

By an “isolated polypeptide” is meant a P450 polypeptide (for example, a CYP71D20 (SEQ ID NO:1), CYP71D21 (SEQ ID NO:3), CYP73A27 (SEQ ID NO:5), CYP73A28 (SEQ ID NO:7), or CYP92A5 (SEQ ID NO:11) polypeptide) that has been separated from components that naturally accompany it. Typically, the polypeptide is isolated when it is at least 60%, preferably at least 75%, and most preferably at least 90%, and most preferably at least 99%, by weight, a P450 polypeptide. An isolated P450 polypeptide may be obtained, for example, by extraction from a natural source (for example, a plant cell); by expression of a recombinant nucleic acid encoding a P450 polypeptide; or by chemically synthesizing the protein. Purity can be measured by any appropriate method, for example, column chromatography, polyacrylamide gel electrophoresis, or by HPLC analysis.

By “derived from” or “obtained from” is meant isolated from or having the sequence of a naturally-occurring sequence (e.g., CDNA, genomic DNA, synthetic, or combination thereof).

By “isolated nucleic acid molecule” is meant a nucleic acid molecule, e.g., a DNA molecule, that is free of the nucleic acid sequence(s) which, in the naturally-occurring genome of the organism from which the nucleic acid molecule of the invention is derived, flank the nucleic acid molecule. The term therefore includes, for example, a recombinant DNA that is incorporated into a vector; into an autonomously replicating plasmid or virus; or into the genomic DNA of a prokaryote or eukaryote; or that exists as a separate molecule (for example, a cDNA or a genomic or cDNA fragment produced by PCR or restriction endonuclease digestion) independent of other sequences. The term “isolated nucleic acid molecule” also includes a recombinant DNA which is part of a hybrid gene encoding additional polypeptide sequences.

By “specifically hybridizes” is meant that a nucleic acid sequence is capable of hybridizing to a DNA sequence at least under low stringency conditions, and preferably under high stringency conditions. For example, high stringency conditions may include hybridization at approximately 42°C in about 50% formamide, 0.1 mg/ml sheared salmon sperm DNA, 1% SDS, 2xSSC, 10% Dextran sulfate, a first wash at approximately 65°C in about 2xSSC, 1% SDS, followed by a second wash at approximately 65°C in about 0.1xSSC. Alternatively high stringency conditions may include hybridization at approximately 42°C in about 50% formamide, 0.1
mg/ml sheared salmon sperm DNA, 0.5% SDS, 5×SSPE, 1×Denhardt’s, followed by two washes at room temperature in 2×SSC, 0.1% SDS, and two washes at between 55-60°C in 0.2×SSC, 0.1% SDS. Reducing the stringency of the hybridization conditions may involve lowering the wash temperature and/or washing at a higher concentration of salt. For example, low stringency conditions may include washing in 2×SSC, 0.1% SDS at 40°C.

By “transformed cell” is meant a cell into which (or into an ancestor of which) has been introduced, by means of recombinant DNA techniques, a DNA molecule encoding (as used herein) a P450 polypeptide.

By “positioned for expression” is meant that the DNA molecule is positioned adjacent to a DNA sequence which directs transcription and translation of the sequence (i.e., facilitates the production of, for example, a P450 polypeptide, a recombinant protein, or an RNA molecule).

By “reporter gene” is meant a gene whose expression may be assayed; such genes include, without limitation, beta-glucuronidase (GUS), luciferase, chloramphenicol acetyltransferase (CAT), green fluorescent protein (GFP), beta-galactosidase, herbicide resistant genes, and antibiotic resistance genes.

By “expression control region” is meant any minimal sequence sufficient to direct transcription. Included in the invention are promoter elements that are sufficient to render promoter-dependent gene expression controllable for cell-, tissue-, or organ-specific gene expression, or elements that are inducible by external signals or agents (for example, light-, pathogen-, wound-, stress-, or hormone-inducible elements or chemical inducers such as salicylic acid (SA) or 2,2-dichloroisonicotinic acid (INA)); such elements may be located in the 5’ or 3’ regions of the native gene or engineered into a transgene construct.

By “operably linked” is meant that a gene and a regulatory sequence(s) are connected in such a way as to permit gene expression when the appropriate molecules (for example, transcriptional activator proteins) are bound to the regulatory sequence(s).

By “plant cell” is meant any self-propagating cell bounded by a semi-permeable membrane and typically is one containing a plastid. Such a cell also requires a cell wall if further propagation is desired. Plant cells, as used herein includes, without limitation, algae, cyanobacteria, seeds, suspension cultures, embryos, meristemetic regions, callus tissue, leaves, roots, shoots, gametophytes, sporophytes, pollen, and microspores.

By “plant component” is meant a part, segment, or organ obtained from an intact plant or plant cell. Exemplary plant components include, without limitation, somatic embryos, leaves, stems, roots, flowers, tendrils, fruits, scions, and rootstocks.

By “transgene” is meant any piece of DNA which is inserted by artifice into a cell and typically becomes part of the genome, for example, the nuclear or plastidic genome, of the organism which develops from that cell. Such a transgene may include a gene which is partly or entirely heterologous (i.e., foreign) to the transgenic organism, or may represent a gene homologous to an endogenous gene of the organism.

By “transgenic” is meant any cell which includes a DNA sequence which is inserted by artifice into a cell and becomes part of the genome of the organism which develops from that cell. As used herein, the transgenic organisms are generally transgenic plants and the DNA (transgene) is inserted by artifice into the nuclear or plastidic genome. A transgenic plant according to the invention may contain one or more engineered traits.

By “pathogen” is meant an organism whose infection of viable plant tissue elicits a disease response in the plant tissue. Such pathogens include, without limitation, bacteria, mycoplasmas, fungi, insects, nematodes, viruses, and viroids. Plant diseases caused by these pathogens are described in Chapters 11-16 of Agrics, Plant Pathology, 3rd ed., Academic Press, Inc., New York, 1988.

By “increased level of resistance” is meant a greater level of resistance to a disease-causing pathogen in a transgenic plant (or cell or seed thereof) of the invention than the level of resistance relative to a control plant (for example, a non-transgenic plant). In preferred embodiments, the level of resistance in a transgenic plant of the invention is at least 20% (and preferably 30% or 40%) greater than the resistance of a control plant. In other preferred embodiments, the level of resistance to a disease-causing pathogen is 50% greater, 60% greater, and more preferably even 75% or 90% greater than a control plant; with up to 100% above the level of resistance as compared to a control plant being most preferred. The level of resistance is measured using conventional methods. For example, the level of resistance to a pathogen may be determined by comparing physical features and characteristics (for example, plant height and weight, or by comparing disease symptoms, for example, delayed lesion development, reduced lesion size, leaf wilting and curling, water-soaked spots, and discoloration of cells) of transgenic plants.

By “purified antibody” is meant antibody which is at least 60%, by weight, free from proteins and naturally-occurring organic molecules with which it is naturally associated. Preferably, the preparation is at least 75%, more preferably 90%, and most preferably at least 99%, by weight, antibody, for example, an acquired resistance polypeptide-specific antibody. A purified P450 antibody may be obtained, for example, by affinity chromatography using a recombinantly-produced P450 polypeptide and standard techniques.

By “specifically binds” is meant an antibody which recognizes and binds a P450 protein but which does not substantially recognize and bind other molecules in a sample, for example, a biological sample, which naturally includes a P450 protein such as CYP71D20, CYP71D21, CYP73A27, CYP73A28, or CYP92A5. Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic diagram of a proposed alternative pathway for the biosynthesis of capsidiol in elicitor-treated Nicotiana tabacum cells. 5-epi-aristolochene is synthesized from FPP by the action of a sesquiterpene cyclase, 5-epi-aristolochene synthase (EAS), and is subsequently hydroxylated at C1 and C3 to form capsidiol.

FIG. 2 is a graph showing an induction time course for sesquiterpene cyclase enzyme activity and sesquiterpene hydroxylase activity in cellulase-treated cell cultures. Sesquiterpene cyclase (5-epi-aristolochene synthase, EAS) enzyme activity was determined in extracts prepared from control (open squares) and elicitor-treated (closed squares) cells collected at the indicated time points. Sesquiterpene hydroxylase activity was determined using an indirect assay for control (open circles) and elicitor-treated (closed circles) cells. Cell cultures were incubated with [3H]-5-epi-aristolochene for 3 hours ending at the indicated time points before quantifying the incorporation of radioactivity into

FIG. 3 is a series of graphs showing the dose dependent inhibition of 5-epi-aristolochene hydroxylase activity by anecymidol and ketoconazole. Cells cultures were incubated in the presence of cellulase (0.5 μg/ml) plus the indicated concentrations of anecymidol (A) or ketoconazole (B) for 12 hours prior to measuring the in vivo 5-epi-aristolochene hydroxylase activity in the cell suspension cultures (squares), or the EAS enzyme activity in extracts prepared from the collected cells (triangles). The in vitro activity of a purified EAS preparation (Back and Chappell, J. Biol. Chem. 270:7375-7381, 1995) was also measured at the indicated inhibitor concentrations as an additional test for non-specific effects of these inhibitors (circles).

FIG. 4A is a schematic diagram of the primary structure of a generalized cytochrome P450 with conserved domains used for the design of PCR primers highlighted (SEQ ID NO:26-29).

FIG. 4B is a list of the degenerate P450-specific primers (SEQ ID NO:30-36) that were used in various combinations with vector specific primers in the amplification of cytochrome P450 cDNA fragments.

FIG. 4C is a scanned image of an ethidium bromide-stained agarose gel showing the PCR products amplified from a directional cDNA library prepared with mRNA isolated from elicitor-treated cells using the degenerate primer GRRXCP (A/G) for (SEQ ID NO:35) and the T vector-specific primer (SEQ ID NO:37). The T3 vector-specific primer is also shown (SEQ ID NO:38).

FIG. 5 is a series of Northern blots showing the induction time course for CYP71D, CYP73A, CYP82E, CYP92A, and EAS transcript accumulation in elicitor treated cells. Total RNA was extracted from tobacco suspension cells incubated with the cellulase elicitor for the indicated durations. Size fractionated by agarose gel electrophoresis under denaturing conditions, and transferred to a nylon membrane before probing with the respective full-length cDNAs. The uniformity of sample loading was verified by ethidium bromide staining of ribosomal RNA (Loading control).

FIG. 6 is a series of graphs showing carbon monoxide (CO) difference spectra of the microsomal fraction isolated from yeast expressing the CYP92A5 (A) and CYP71D20 (B) cDNAs. Expression of the respective plasmid constructs engineered into the yeast (WAT11) cells was induced by a galactose treatment, followed by isolation of microsomal preparations. The difference absorption spectra of microsomes incubated in the presence (solid lines) and absence (broken lines) of carbon monoxide was determined.

FIG. 7 is a series of gas chromatograms of the reaction products formed upon incubation of microsomes isolated from WAT1 yeast cells containing the CYP71D20 expression construct (A and C) or vector control DNA (B and D) with sesquiterpene substrates. Microsomes isolated from the indicated yeast lines were incubated with 5-epi-aristolochene (A and B) or 1-deoxycapsidiol (C and D) in the presence (solid lines) or absence (dashed lines) of NADPH. The identities of 5-epi-aristolochene, 1-deoxycapsidiol, and capsidiol were verified by mass spectrometry.

FIGS. 8A-D provide a sequence comparison of the amino acid sequence of Nicotiana tabacum 5-epi-aristolochene (sesquiterpene) hydroxylase NcCYP71D20 (SEQ ID NO:1) with other plant terpene hydroxylases (SEQ ID NO:39-43). NcCYP71A5v1 (GenBank accession number CAA70575) catalyzes the mono-hydroxylation of nerol and geraniol, linear monoterpenes, while PaCYP71A1 (A35867) catalyzes the epoxidation of these substrates (Hallahan et al., Biochim. Biophys. Acta. 1201:94-100, 1994). MsCYP71D18 (AAD44150) and MtCYP71D13 (AAD44151) catalyze the mono-hydroxylation at C6 and C3 of limonene, a cyclic monoterpane, respectively (Lupien et al., Arch. Biochem. Biophys. 368:181-192, 1999). AtCYP71A3 (AAC39505) encodes for kauren oxidase, which catalyzes a 3-step reaction including a hydroxylation followed by oxidation of a diterpene (Hellwell et al., Plant Physiol. 119:507-510, 1999). Shown are sequences from Mentha piperita (MpcYP71D13; SEQ ID NO:39), Mentha spicata (MscCYP71D18; SEQ ID NO:40), Nepeta racemosa (NrCYP71A5v1; SEQ ID NO:41), Nicotiana tabacum (NcCYP71D20; SEQ ID NO:41), Persea americana (PaCYP71A1; SEQ ID NO:42), and Arabidopsis thaliana (CYP701A3; SEQ ID NO:43). Conserved residues are shaded.

DETAILED DESCRIPTION

Capsidiol is a bicyclic, dihydroxylated sesquiterpene produced by several Solanaceae species in response to a variety of environmental stimuli. It is the primary antimicrobial compound produced by Nicotiana tabacum in response to fungal elicitation, and it is formed via the isoprenoid pathway from 5-epi-aristolochene. Much of the biosynthetic pathway for the formation of this compound has been elucidated, except for the enzyme(s) responsible for the conversion of the allylic sesquiterpene 5-epi-aristolochene to its dihydroxylated form, capsidiol.

Accordingly, in an in vitro assay for 5-epi-aristolochene hydroxylase activity was developed and used to demonstrate a dose dependent inhibition of activity by anecymidol and ketoconazole, two well-characterized inhibitors of cytochrome P450 enzymes. Using degenerate oligonucleotide primers designed to the well-conserved domains found within most P450 enzymes, including the heme binding domain, cDNA fragments representing four distinct P450 families (CYP71, CYP73, CYP82, and CYP92) were amplified from a cDNA library prepared from mRNA from elicitor-treated cells using PCR. The PCR fragments were subsequently used to isolate full-length cDNAs (CYP71D20 (SEQ ID NO:2) and D21 (SEQ ID NO:4), CYP73A27 (SEQ ID NO:6) and A28 (SEQ ID NO:8), CYP82E1 (SEQ ID NO:10), and CYP92A5 (SEQ ID NO:12)), and these in turn were used to demonstrate that the corresponding mRNAs were all induced in elicitor-treated cells, albeit with different induction patterns.

EXAMPLES

There now follows a description of the cloning of several P450s from Nicotiana tabacum. These examples are provided for the purpose of illustrating the invention, and are not to be considered as limiting.

Inhibition of the 5-epi-aristolochene to Capsidiol Conversion by P450 Antagonists

Using an indirect assay, a detailed induction time course of SEA activity in elicitor-induced cell cultures was determined relative to that of EAS activity (FIG. 2), the well-characterized sesquiterpene cyclase activity that catalyzes the formation of 5-epi-aristolochene from FPP (FIG. 1). Using assays for EAS and SEA, EAS activity is not detectable in control cell cultures, but is induced significantly within 3 hours and reaches its maximal level within 15 to 18 hours of elicitor-treatment. Similar to the EAS enzyme activity, SEA1 activity was negligible in control cell cultures. Nonetheless,
after an apparent lag phase of 8 hours, a rapid induction of hydroxylase activity was observed 10 to 15 hours post elicitor addition to the cell cultures, reaching a maximum by 18 hours followed by a rather gradual decline of 10 to 20% over the next 8 hours.

Tobacco cell suspension cultures treated with cellulase plus varying concentrations of ancyemid or ketoconazole were pre-incubated for 12 hours before measuring the cells’ ability to convert exogenous supplied [1H] labeled 5-epi-aristolochene to radiolabeled capsidol during a subsequent 3 hour incubation period (Fig. 3). Apparent activity of SEAH was inhibited in a dose-dependent manner with approximately 50% inhibition by either 25μM ancyemid or ketoconazole, and more than 80% by 75μM ancyemid and 95% by 100μM ketoconazole (Fig. 3A and B). Importantly, neither the in vitro activity of recombinant EAs nor the induction of EAS in the elicitor-treated cell cultures was significantly affected by ancyemid at concentrations as high as 100μM (Fig. 3A). Ketoconazole also does not appear to affect the in vitro activity of EAs. However, the inducibility of cyclase activity in elicitor-treated cell extracts was inhibited by ketoconazole at concentrations above 50μM (Fig. 3B). Therefore, the specificity of ketoconazole as an inhibitor of P450 type reactions should be assessed at or below a concentration of 50μM under these experimental conditions.

Isolation of Elicitor-inducible Cytochrome P450 cDNAs

A two-step approach for the isolation of candidate P450 cDNAs was followed. A PCR strategy was first employed using a directional cDNA library prepared against mRNA isolated from elicitor-induced cells as the template and degenerate PCR primers (Fig. 4). Sequence alignments of cytochrome P450s from multiple families across kingdoms were used to identify conserved regions to which a series of degenerate primers were prepared (Figs. 4A and B). In cloning experiments, 450 to 550 by products were expected from reactions utilizing the primer prepared to the heme-binding domain (GRRXCP(A/G)(SEQ ID NO:27 and 28) and the 17 vector primer (Fig. 4C)). The mixtures of reaction products were shotgun cloned, and approximately 100 of the cloned PCR fragments were sequenced. About half of the sequenced DNA contained signature sequences typical of P450 enzymes as revealed by BlastX database searches, and these corresponded to typical plant P450 family members of the CYP7, CYP93, CYP92 and CYP82 classes. Each of these PCR fragments was isolated multiple times in separate experiments. In addition, we isolated full-length cDNAs for these P450 family members. Table I compares the similarity and identity of the full-length cDNAs of P450 family members with those of their nearest family member in the GenBank database. In addition, Fig. 8 shows an amino acid alignment of several terpene cytochrome P450s. Alignments were performed using the algorithm of the MACVECTOR software suite.

TABLE 1

<table>
<thead>
<tr>
<th>Cytochrome P450 cDNA clone</th>
<th>Nearest relative/ accession number</th>
<th>% Identity</th>
<th>% Similarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP7D20</td>
<td>CYP7D7 (S. cacao)</td>
<td>76.5</td>
<td>88.8</td>
</tr>
<tr>
<td>CYP7D21</td>
<td>Gen EmBL U68435</td>
<td>76.3</td>
<td>88.8</td>
</tr>
<tr>
<td>CYP7A27</td>
<td>CYP7A15 (F. vulgaris)</td>
<td>79.4</td>
<td>92.6</td>
</tr>
</tbody>
</table>

The cloned fragments were used in a second step to isolate full-length cDNAs from the cDNA library. Screening the cDNA library by hybridization with the CYP71 and CYP73 gene fragments yielded four full-length cDNAs, two CYP71 Ds and two CYP73As. The former clones were designated CYP71D20 and CYP71D21, and the latter were designated CYP73A27 and CYP73A28. The other two cDNA fragments corresponded to tobacco cDNAs already found in the GenBank database, CYP82D1 and CYP92A3. These two cDNAs were cloned using specific primers designed with the help of the available sequence information to amplify the full-length cDNA.

Induction of Cytochrome P450 mRNAs in Elicitor-treated Cells

To correlate a biochemical role for P450s in sesquiterpene metabolism, RNA blot analyses were used to determine the steady-state levels of the mRNAs coding for all four of the cytochrome P450 clones and EAs in control and elicitor-treated cells (Fig. 5). The mRNAs for all four of the P450s were rapidly and transiently induced with slightly different time courses relative to one another and to the EAs mRNA. CYP73A27 mRNA, for instance, displayed an induction pattern similar to that of EAs with the maximum mRNA level occurring 9 to 12 hours after elicitation. While the CYP73A27 mRNA remained high throughout the duration of the experiment, the CYP92A27 mRNA was negligible in cells 24 hours after elicitor-treatment. In contrast, the CYP71D2 mRNA was more rapidly induced than the EAs mRNA, reached its maximum 6 to 9 hours after elicitation, and was declining by 12 hours when the EAs mRNA level was still very high.

Functional Identification of CYP71 D20 as 5-Epi-aristolochene Hydroxylase

To assign functional identity to the various P450 cDNAs, full-length cDNAs for CYP71D20, CYP82D1 and CYP92A5 were inserted into the yeast expression vector pYeDP60 (Urban et al., Biochemistry 72:463-472, 1990; Pompon et al., Methods Enzymol. 272:51-64, 1996) and the expression of each in WAT11, a yeast line containing an integrated Arabidopsis thaliana cytochrome reductase gene (Pompon et al., Methods Enzymol. 272:51-64, 1996; Urban et al., J. Biol. Chem. 272: 19176-19186, 1997), was determined. Engineering the CYP73A27 cDNA required an extra modification because of an unusually long N-terminus with several hydrophilic residues that may interfere with proper intracellular targeting (Nedelkina et al., Plant Mol. Biol. 39:1079-1090, 1999). This unusual leader sequence therefore was replaced with the membrane anchoring sequence of CYP73A1, a cinnamate 4-hydroxylase previously demonstrated to express well in yeast (Führendorf and Dixon, Arch. Biochem. Biophys. 305: 509-515, 1993; Pompon et al., Methods Enzymol. 272:51-64, 1996). Expression of all these cDNAs was under the control of the glucose-repressible, galactose-inducible GAL10 CYC1 promoter (Guarente et al., Proc. Natl. Acad. Sci.
In vivo 5-epi-ariistolochene Hydroxylase Assay and Inhibition Studies

5-epi-ariistolochene hydroxylase-activity was measured as the incorporation of [3H]-5-epi-ariistolochene into extracellular capsidiol by intact cells. [3H]-5-epi-ariistolochene was produced by incubating an excess of [3H]-farnesyl diphostate (1 μM, 20.5 Ci/mmol) with recombinant 5-epi-ariistolochene synthase (Buck et al., Arch. Biochem. Biophys. 315:527-532, 1994; Rising et al., J. Am. Chem. Soc. 122:1861-1866, 2000). The hexane extractable radioactivity from reactions was treated with a small amount of silica to remove any farnesol or residual FBP before quantifying the yield of radioactive 5-epi-ariistolochene by liquid scintillation counting.

The hexane solvent was removed under a gentle stream of N2 gas, and the dried residue was re-dissolved in acetone. Control and elicitor-treated cells were then incubated with [3H]-5-epi-ariistolochene (approximately 100,000 dpm at 2.5 nM) for 3 hour periods at various points during an induction time course before collecting the cell and media samples.

Detection and quantification of capsidiol in the extracellular culture media was performed as reported previously (Chappell et al., Phytochemistry 26:2259-2260, 1987), and the amount of radioactivity incorporated into capsidiol was determined. For these determinations, samples were separated by TLC, and the zones corresponding to capsidiol were scraped from the plate for scintillation counting.

Inhibition studies were performed by the addition of the P450 inhibitors ancamidol (Coolbaugh et al., Plant Physiol. 62:571-576, 1978; Hoshino et al., Phytochemistry 38:609-613, 1995) and ketoconazole (Hoshino et al., Phytochemistry 38:609-613, 1995; Rademacher, Annu. Rev. Plant Physiol. Plant Mol. Biol. 51:501-531, 2000) directly to the cell cultures or enzyme assay mix. Cell cultures were incubated in the presence of ancamidol (0.5 μg/ml) and indicated concentrations of ancamidol or ketoconazole for 12 hours prior to the addition of [3H]-5-epi-ariistolochene. After a further 3 hour incubation period, the cells and media were collected.

The amount of radioactivity incorporated into extracellular capsidiol was determined as described above. To evaluate secondary effects of these inhibitors, the level of inducible sesquiterpene cyclase activity in the collected cells was determined according to Vogel et al. (Plant Physiol. 93:182-187, 1990), as well as in vitro assays with purified recombinant EAS (Back et al., Arch. Biochem. Biophys. 315:527-532, 1994) incubated with the indicated concentrations of ancamidol and ketoconazole.

All experiments were replicated in several independent trials. While the absolute values presented may have varied between experiments by as much as 50%, the trends and time courses were consistent throughout.

Construction of an Elicitor-induced cDNA Library

Cell cultures were incubated with fungal elicitor (0.5 μg cellulase/ml) for 6 hours before collecting the cells by filtration. The cells were kept frozen at -80°C until total RNA was extracted from them using Trizol (Life Technologies, Rockville, Md.) according to the manufacturer’s instructions. Poly (A)"RNA was purified by two rounds of oligo (dT) cellulose column chromatography (Life Technologies, Rockville, Md.). cDNA synthesis and library construction were subsequently carried out using the UNI-ZAP XR library kit (Stratagene, La Jolla, Calif.), according to manufacturer’s instructions.

PCR Cloning Strategy

Cytochrome P450 cDNA fragments were amplified from the elicitor-induced cDNA library using various combinations of degenerate forward and reverse primers with the vector-specific T3 and T7 primers. The template DNA was
prepared from a 500 µl aliquot of the elicitor-induced cDNA library (3 x10^6 pfu/µl) by heat denaturation at 70°C for 10 minutes, followed by phenol/chloroform extraction, ethanol precipitation and resuspension in 500 µl of sterile, deionized water. Amplification reactions were performed in 50 µl volumes containing 50 mM KCl; 10 mM Tris-HCl, pH 8.8; 1.5 mM MgCl₂; 200 µM of each dNTP; 2 µl template DNA; 20 pmol each of forward and reverse primer; and 1 unit Taq Polymerase (Life Technologies, Rockville, Md.). Reactions were preheated at 94°C for 2 minutes, followed by thirty-five cycles of denaturing at 94°C for 1 minute, annealing at 50°C for 1 minute 30 seconds, and polymerization at 72°C for 2 minutes. The reactions were completed by a 10-minute extension at 72°C. Aliquots of the reaction products were examined for DNA products by agarose gel fractionation, and ligated directly into the pGEM-T Easy vector (Promega, Madison, Wis.). Resulting recombinant plasmids containing insert DNA within the expected size range were sequenced using 17 and SP6 primers.

DNA Sequencing

All the DNA sequencing reactions were performed using the BIGDYETM Terminator Cycle sequencing kit (Perkin-Elmer, Wellesley, Mass.) with the sequences being read on an automated ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, Calif.). Computer assessment of the DNA sequence information was performed using the MACVECTOR (Oxford Molecular, Madison, Wis.) software package.

cDNA Library Screening The cDNA library was screened with digoxigenin labeled probes. A 258 bp DNA fragment amplified from the pGEM-deg6.4 clone using generic specific forward (5'-GGCGGAGAATTCTGGAAATGTC- CATTTCTTAG-3' (SEQ ID NO:13)) and reverse (5'- GTACATAGTAGTGAACATGAC-3' (SEQ ID NO:14)) primers; and a 374 bp DNA fragment amplified from the pBKS-CYP3B.843 clone with specific forward (5'-GGTGGTTAGAACTGACG-3' (SEQ ID NO:15)) and reverse (5'- TATGCGACATCAAGGCAGGAGC-3' (SEQ ID NO:16)) primers, were used to screen for CYP71Ds. The probes were labeled with digoxigenin-11-UTP using the PCR DIG Labeling Mix (Roche Molecular Biochemicals, Indianapolis, Ind.), hybridized to plasmid lift of the cDNA library plated at approximately 10,000 PFUs per 150 mm plate, and was hybridization detected with the DIG detection system according to the manufacturer's instructions (Roche Molecular Biochemicals, Indianapolis, Ind.). Plasmid exo plastin strong hybridization were plaque purified, auto-subcloned to their plasmid forms according to the manufacturer's recommendations (Stratagene, La Jolla, Calif.), and then subjected to DNA sequencing as described above.

RNA Analysis

RNA gel blot analysis was carried out using 10 µg aliquots of total RNA. RNA samples were heat-denatured at 70°C for 15 minutes in sample buffer (1x MOPS, 50% formamide, 16% formaldehyde, 30% glycerol, and 3% ethidium bromide), and size fractionated on a 1.2% agarose gel containing 1x MOPS and 18.1% formaldehyde. Uniformity of sample loading was determined by visual inspection of the gel for RNA bands. The RNAs were then transferred to a Zeta Probe nylon membrane (Bio-Rad Laboratories, Hercules, Calif.) and hybridized according to the manufacturer's recommendations. Full-length cDNA probes were labeled with [32P]-dCTP (PRIME-IT Kit, Stratagene, La Jolla, Calif.) prior to hybridization. After hybridization, the membranes were washed in 2x SSC/0.1% SDS once at room temperature followed by sequential washes in 0.2x SSC/0.1% SDS at 42°C, and 65°C. Hybridization was detected with a Phosphoimager (Molecular Dynamics, model 445 SI).

Construction of Yeast Expression Vectors

The coding regions of the P450 cDNAs were cloned into the pYeDP60 expression vector (Urban et al., J. Biol. Chem. 272:19176-19185, 1997; Pompon et al., Methods Enzymol. 272:51-64, 1996). Appropriate BamHI, EcoRI, and Sst restriction sites (underlined) were introduced via PCR primers containing these sequences either upstream of the translation start site (ATG) or downstream of the stop codon (TAA or TGA). The primers used to amplify the CYP71D20 cDNA were 5'-GGGGGAATCC ATGCAATTCCTGACCGTTGCTGCC-3' (SEQ ID NO:17) and 5'-GGGGGAATTC TTAATCCAGAGAATGTTGAAGC-3' (SEQ ID NO:18) for the CYP71D20 cDNA 5'-CCCGAGATCC ATGAT1CATCCTCAGTCTTTTCCTGCC-3' (SEQ ID NO:19) and 5'-GGGGGAATTC TCAATTGATAGACCGTGGAGG-3' (SEQ ID NO:20) and for the CYP71D20 cDNA 5'-CCCGAGATCC ATGCAATTCCTGACCGTTGCTGCC-3' (SEQ ID NO:21) and 5'-GGGGGAATTC TCAATTGATAGACCGTGGAGG-3' (SEQ ID NO:20).

Two long, overlapping (stabilized) primers 5'-GCCATATGCGGCGAATCAATATTTCGCA-3' and 5'- GCCATATGCGGCGAATCAATATTTCGCA-3' were used for the construction of the membrane anchoring segment of CYP73A1 (GenEMBL Z17369) up to the hinge region were used for the modification of the membrane anchoring segment of CYP73A27 to avoid possible problems with intracellular targeting due to the unusual N-terminus (Nedelkina et al., 1999); the reverse primer used for both amplifications was 5'-GGGGGAATTC TTAATCCAGAGAATGTTGAAGC-3' (SEQ ID NO:23) and CYP71D20 and CYP73A27 were amplified using full-length cDNA templates, whereas CYP71D20 and CYP72A5 were amplified directly from the cDNA library template. Amplifications were performed in 50 µl reactions containing 1xPx amplification buffer; 1 mM MgSO₄, 300 µM of each dNTP; 10 ng template DNA; 20 pmol each of forward and reverse primer; and 1.25 units PLATINUM® Pfx Polymerase (Life Technologies, Rockville, Md.). Reactions were preheated at 94°C for 2 minutes, followed by thirty-five cycles of denaturing at 94°C, for 15 seconds, annealing at 55°C for 30 seconds, and elongating at 68°C for 1.5 minutes. PCR products were ligated into the pGEM-T EASY vector (Promega, Madison, Wis.) and subcloned into the pYeDP60 vector. The resulting constructs were validated by a combination of PCR and DNA sequencing.

Yeast Expression Studies

Yeast expression plasmids were transformed into the yeast strain W303-1B strain (MAT a; ade 2-1; his 3-11; leu 2-3;112; ura 3-1; can6; cyr+), provided by Dr. P. Urban (Centre de Génétique Moléculaire, CNRS, Gif-sur-Yvette, France). The endogenous NADPH-cytchrome P450 reductase (CPR1) locus has been replaced with ATR1, a NADPH-cytchrome P450 reductase from Arabidopsis thaliana (Pompon et al., Methods Enzymol. 272:51-64, 1996; Urban et al., J. Biol. Chem. 272:19176-19185, 1997), in the WAT11 line. Yeast was grown overnight in a 30°C shaker in YPAD (1 g/l yeast extract; 1 g/l peptone; 20 g/l glucose; 200 mg/l adenine) liquid media. Cultures were harvested at an A₆₀₀ between 0.5 and 1.5. Cells were collected by centrifugation at 2,500 xg for 5 minutes at 4°C, and resuspended in ice-cold, sterile dH₂O. Cells were pelleted again as above and resuspended in 1M sorbitol. Forty µl of yeast suspension was mixed with 0.5 to 1
µg plasmid DNA (in 5 µl dH2O) in a pre-chilled 0.5 ml tube, and transferred to a chilled vial with a 0.2 cm electrode gap. One pulse at 1.5 kV, 25 µF, and 200 Ohms was applied by an Eppendorf Electrophorator (Model 2510). A mixture of 500 µl of YPAD/1M sorbitol was immediately added to the electroporated cells. Cells were allowed to recover at 30°C, for 1 h, then spread onto SGI plates (1 g/l bacterocasaminol acids; 7 g/l yeast nitrogen base; 20 g/l glucose; 20 mg/l tryptophan; and 20 g/l agar). Transformed colonies appeared after 3 to 6 days of incubation at 30°C. Recombinant plasmids were confirmed by PCR assays performed directly on randomly selected yeast colonies.

For expression studies, one colony was added to SGI media (1 g/l bacterocasaminol acids; 7 g/l yeast nitrogen base; 20 g/l glucose; and 20 mg/l tryptophan) and grown at 30°C for approximately 24 hours. An aliquot of this culture was diluted 1:50 into 250 ml of YPGE (10 g/l bacterocasaminol; 10 g/l yeast extract; 5 g/l glucose; and 3% ethanol by volume) and the cells were grown until all glucose was consumed. The absence of glucose was determined by placing a 200 µl aliquot of culture into a 1.5 ml tube, inserting a DiaStix urinalysis reagent strip (Bayer, Elkhart, IN) for 30 seconds, and observing colorimetric changes indicating glucose levels. Induction was initiated by the addition of 5 grams of galactose (final concentration of 2%). The cultures were maintained at 30°C for an additional 16 hours before collecting the cells by centrifugation at 7,000 x g for 10 minutes. The pellets were washed with 100 ml of TES buffer (50 mM Tris-Cl, pH 7.5; 1 mM EDTA; 0.6 M sorbitol). The cells were centrifuged as above, resuspended in 100 ml of TES-M (TES supplemented with 10 mM 2-mercaptoethanol), and allowed to incubate at room temperature for 10 minutes. The yeast cells were centrifuged again at 7,000 x g for 10 minutes, and the pellet was resuspended in 2.5 ml extraction buffer (180 mM bovine serum albumin, 1.25 M NaCl, 2 M 2-mercaptoethanol; 1 mM N-phenylmethyl-p-tolyl sulfoxide, all dissolved in TES). Glass beads (0.5 mm in diameter, Biospec Products, Inc., Bartlesville, Okla.) were added until the surface of the cell suspension. Cell walls were disrupted manually by hand shaking in a cold room for 10 min at 30°C separated by 30 second intervals. Cell extracts were transferred to a 50 ml centrifuge tube, the glass beads were washed three times with 5 ml of extraction buffer, and the washes were pooled with the original cell extract. Microsomes were prepared by differential centrifugation at 10,000 g for 10 minutes at 4°C to remove cellular debris, followed by centrifugation at 100,000 x g for 70 minutes at 4°C, and microsomal pellets were resuspended in 1.5 ml TEG-M buffer (50 mM Tris-Cl, pH 7.5; 1 mM EDTA; 20% glycerol; and 1.5 mM 2-mercaptoethanol) and stored frozen at -80°C until further assayed.

CO Difference Spectra
Fe(III) vs. Fe(II) difference spectroscopy (Omoruyi and Sato, J. Biol. Chem. 239:2370-2378, 1964) was performed using 0.4 ml of microsomes suspended in 1.6 ml of 50 mM Tris-Cl, pH 7.5; 1 mM EDTA; and 20% glycerol. A small amount of the reducing agent, sodium dithionite, was added, and the mixture was distributed between two cuvettes. A baseline was recorded between 400 and 500 nm on a Perkin Elmer Lambda 18 UV/visible spectrophotometer. CO was then bubbled into the sample cuvette for 1 minute, and the difference spectrum recorded again. The amount of functional P450 was estimated based on an absorbance coefficient of 91 mM⁻¹ ·cm⁻¹.

5-Epi-aristolochene-1,3-hydroxylase Assays
5-Epi-aristolochene-1,3-hydroxylase assays were performed in 0.5 ml polyethylene tubes in 100 mM volumes. 5-epi-aristolochene or 1-deoxyxyspidol dissolved in hexane was added to the tube, and the organic solvent was removed by incubation of the open tube at 30°C. 5-epi-aristolochene and 1-deoxyxyspidol were resuspended in 2 µl dimethyl sulfoxide before adding the reaction mixture. Reactions were carried out in 100 mM Tris-Cl, pH 7.5, to which microsomal protein was added to a final concentration of 1 mg/ml. Reactions were initiated by the addition of 2 mM NADPH. The final concentration of 5-epi-aristolochene and 1-deoxyxyspidol in these assays varied from 20 to 50 µM. After incubations for variable lengths of time at 30°C, the reactions were extracted with two volumes of ethyl acetate. The organic extracts were concentrated and evaluated by GC and GC-MS along with standards of 5-epi-aristolochene (Whitehead et al., Phytochemistry 28:775-779, 1989; Rising et al., J. Am. Chem. Soc. 122:1861-1866, 2000), 1-deoxyxyspidol (Whitehead et al., Phytochemistry 29:479-182, 1990), and capsidol (Whitehead et al., Phytochemistry 26:1367-1369, 1987; Miliot et al., Phytochemistry 30:2171-2173, 1991). GC analysis was routinely performed with an HP5890 GC equipped with a Hewlett-Packard HP-5 capillary column (30 m x 0.25 mm, 0.25 gm phase thickness) and FID as described previously (Rising et al., J. Am. Chem. Soc. 122:1861-1866, 2000). GC-MS analysis was performed at the University of Kentucky Mass Spectrometry Facility using a Varian 3400 gas chromatograph and a Finnigan INCOS 50 quadrupole mass selective detector. The GC was equipped with a J&W DB-5ms capillary column (15 m x 0.25 mm, 0.25 gm phase thickness) and nH,C as the carrier gas (10 psi). Splitless injections were done at an injection port temperature of 280°C. The column temperature was maintained at 40°C for 1 minute and then increased to 280°C at 10°C per minute. Following separation by the GC column, samples were introduced directly into the electron impact ionization source. Mass spectra were acquired at 70 eV, scanning from 40-440 Da in 1 second.

Production of Cytochrome P450s
Using the standard molecular techniques described herein, the isolation of additional cytochrome P450 coding sequences is readily accomplished. For example, using all or a portion of the amino acid sequence of any of the disclosed P450s, one may readily design P450-specific oligonucleotide probes, including P450 degenerate oligonucleotide probes (i.e., a mixture of all possible coding sequences for a given amino acid sequence). These oligonucleotides may be selected upon the sequence of either DNA strand and any appropriate portion of the P450 nucleotide sequence. General methods for designing and preparing such probes are provided, for example, in Ausubel et al., 2000, Current Protocols in Molecular Biology, Wiley InterScience, New York, and Berger and Kimmel, Guide to Molecular Cloning Techniques, 1987, Academic Press, New York. These oligonucleotides are useful for P450 gene isolation, either through their use as probes capable of hybrizing to a P450 complementary sequence, or as primers for various amplification techniques, for example, polymerase chain reaction (PCR) cloning strategies.

Hybridization techniques and screening procedures are well known to those skilled in the art and are described, for example, in Ausubel et al. (supra); Berger and Kimmel (supra); Chen et al. (Arch. Biochem. Biophys. 324:255, 1995); and Sambrook et al. (Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York). If desired, a combination of different oligonucleotide probes may be used for the screening of a recombinant DNA library. The oligonucleotides may be detectably-labeled using methods known in the art and used to probe filter replicas from a
recombinant DNA library. Recombinant DNA libraries are prepared according to methods well known in the art, for example, as described in Ausubel et al. (supra), or they may be obtained from commercial sources.

As discussed above, P450 oligonucleotides may also be used as primers in a polymerase chain reaction (PCR) amplification cloning strategy. PCR methods are well known in the art and are described, for example, in PCR Technology, Erlich, ed., Stockton Press, London, 1989; PCR Protocols: A Guide to Methods and Applications, Innis et al., eds., Academic Press, Inc., New York, 1990; and Ausubel et al. (supra). Primers are optionally designed to allow cloning of the amplified product into a suitable vector, for example, by including appropriate restriction sites at the 5’ and 3’ ends of the amplified fragment (as described herein). If desired, a P450 gene may be isolated using the PCR “RACE” technique, or Random Amplification of cDNA Ends (see, e.g., Innis et al. (supra)). By this method, oligonucleotide primers based on a P450 sequence are oriented in the 3’ and 5’ directions and are used to generate overlapping PCR fragments. These overlapping 3’- and 5’-end RACE products are combined to produce an intact full-length cDNA. This method is described in Innis et al. (supra) and Frohman et al. (Proc. Natl. Acad. Sci. U.S.A. 85:8998, 1988).

Additional methods for identifying sequences encoding P450s are provided in Maughan et al. (Arch. Biochem. Biophys. 341:104-111, 1997) and Clark et al. (Plant Mol. Biol. 33:875-885, 1997).

Useful P450 sequences may be isolated from any appropriate organism. Confirmation of a sequence’s relatedness to a P450 polypeptide disclosed herein may be accomplished by a variety of conventional methods, for example, by comparing the sequence with a known P450 sequence found in a database. In addition, the activity of any P450 may be evaluated according to any of the techniques described herein.

P450 Polypeptide Expression

P450 polypeptides may be produced by transformation of a suitable host cell with all or part of a P450 DNA (for example, anyone of the P450 cDNAs described herein) in a suitable expression vehicle or with a plasmid construct engineered for increasing the expression of a P450 polypeptide in vivo.

Those skilled in the field of molecular biology will appreciate that any of a wide variety of expression systems may be used to provide the recombinant protein. The precise host cell used is not critical to the invention. The P450 protein may be produced in a prokaryotic host, for example, E. coli TB 1, or in a eukaryotic host, for example, Saccharomyces cerevisiae, insect cells, mammalian cells (for example, COS 1 or NIH 3T3 cells), or any of a number of plant cells including, without limitation, algae, tree species, ornamental species, temperate fruit species, tropical fruit species, vegetable species, legume species, monocots, dicots, or in any plant of commercial or agricultural significance. Particular examples of suitable plant hosts include, but are not limited to, Conifers, Petunia, Tomato, Potato, Tobacco, Gnep, Arabidopsis, Lettuce, Sunflower, Oilseed rape, Flux, Cotton, Sugarbeet, Celery, Soybean, Alfalfa, Medicago, Lotus, Vigna, Cucumber, Carrot, Eggplant, Cauliflower, Horseradish, Morning Glory, Poplar, Walnut, Apple, Asparagus, Grape, Rice, Maize, Milllet, Onion, Barley, Orchard grass, Oat, Rye, Tobacco and Wheat.

Such cells are available from a wide range of sources including the American Type Culture Collection (Rockland, Md.); or from any of a number of seed companies, for example, W. Atlee Burpee Seed Co. (Warminster, Pa.), Park Seed Co. (Greenwood, S.C.), Johnny Seed Co. (Albion, Me.), or Northrup King Seeds (Harstville, S.C.). Descriptions and sources of useful host cells are also found in Vasil I. K., Cell Culture and Somatic Cell Genetics of Plants, Vol 1, II, III; Laboratory Procedures and Their Applications, Academic Press, New York, 1984; Dixon, R. A., Plant Cell Culture—A Practical Approach, IRL Press, Oxford University, 1985; Green et al., Plant Tissue and Cell Culture, Academic Press, New York, 1987; and Gasser and Fraley, Science 244:1293, 1989.

For prokaryotic expression, DNA encoding a P450 polypeptide is carried on a vector operably linked to control signals capable of effecting expression in the prokaryotic host. If desired, the coding sequence may contain, at its 5’ end, a sequence encoding any of the known signal sequences capable of effecting secretion of the expressed protein into the periplasmic space of the host cell, thereby facilitating recovery of the protein and subsequent purification. Prokaryotes frequently used are various strains of E. coli; however, other microbial strains may also be used. Plasmid vectors are used which contain replication origins, selectable markers, and control sequences derived from a species compatible with the microbial host. Examples of such vectors are found in Pouwels et al. (supra) or Ausubel et al. (supra). Commonly used prokaryotic control sequences (also referred to as “regulatory elements”) are defined herein to include promoters for transcription initiation, optionally with an operator, along with ribosome binding site sequences. Promoters commonly used to direct protein expression include the beta-lactamase (penicillinase), the lactose (lac), the tryptophan (Trp) (Goeddel et al., Nucl. Acids Res. 8:4057, 1980), and the tac promoter systems, as well as the lambda-derived Psub1 promoter and N-gene ribosome binding site (Simatake et al., Nature 292:128, 1981).

One particular bacterial expression system for P450 production is the E. coli plasmid expression system (Novagen). According to this expression system, DNA encoding a P450 is inserted into a pET vector in an orientation designed to allow expression. Since the P450 gene is under the control of the T7 regulatory signals, P450 expression is dependent on inducing the expression of T7 RNA polymerase in the host cell. This is typically achieved using host strains which express T7 RNA polymerase in response to IPTG induction. Once produced, recombinant P450 is then isolated according to standard methods known in the art, for example, those described herein.

Another bacterial expression system for P450 production is the pGEX expression system (Pharmacia). This system employs a GST gene fusion system that is designed for high-level expression of a gene or gene fragment as a fusion protein with rapid purification and recovery of the functional gene product. The P450 of interest is fused to the carboxyl terminus of the glutathione S-transferase protein from Schistosoma japonicum and is readily purified from bacterial lysates by affinity chromatography using Glutathione Sepharose 4B. Fusion proteins can be recovered under mild conditions by elution with glutathione. Cleavage of the glutathione S-transferase domain from the fusion protein is facilitated by the presence of recognition sites for site-specific proteases upstream of this domain. For example, proteins expressed in pGEX-2T plasmids may be cleaved with thrombin; those expressed in pGEX-3X may be cleaved with factor Xa.

Other prokaryotic systems useful for expressing eukaryotic P450s are described by Cooper (Mutat. Res. 454:45-52, 2000) and Dong et al. (Arch. Biochem. Biophys. 327:254-259, 1996). In addition, strategies for enhancing the prokaryotic expression of a cytochrome P450 in combination with
cytochrome oxidase are described in Porter et al. (Drug. Metab. Rev. 31:159-174, 1999).


One preferred eukaryotic expression system is the mouse 3T3 fibroblast host cell transfected with a PMAMneo expression vector (Clontech). pMAMneo provides an RSV-LTR enhancer linked to a dexamethasone-inducible MMTV-LTR promoter, an SV40 origin of replication which allows replication in mammalian systems, a selectable neomycin gene, and SV40 splicing and polyadenylation sites. DNA encoding the P450 is inserted into the pMAMneo vector in an orientation designed to allow expression. The recombinant P450 is then isolated as described below. Other preferable host cells which may be used in conjunction with the pMAMneo expression vector include COS cells and CHO cells (ATCC Accession Nos. CRL 1650 and CCL 61, respectively).

Alternatively, if desired, the P450 is produced by a stably transfected mammalian cell line. A number of vectors suitable for stable transfection of mammalian cells are available to the public, e.g., see Poutewels et al. (supra); methods for constructing such cell lines are also publicly available, e.g., in Ausubel et al. (supra). In one example, cDNA encoding the P450 is cloned into an expression vector which includes the dihydrofolate reductase (DHFR) gene. Integration of the plasmid and, therefore, the P450-encoding gene into the host cell chromosome is selected for by inclusion of 0.01-0.30 μg methotrexate in the cell culture medium (as described in Ausubel et al., supra). This dominant selection can be accomplished in most cell types. Recombinant protein expression can be increased by DHFR-mediated amplification of the transfected gene. Methods for selecting cell lines bearing gene amplifications are described in Ausubel et al. (supra); such methods generally involve extended culture in medium containing gradually increasing levels of methotrexate. DHFR-containing expression vectors commonly used for this purpose include pCSEII-DHFr and pAd326SV(A) (described in Ausubel et al., supra). Any of the host cells described above or, preferably, a DHFR-deficient CHO cell line (for example, CHO DHFR cells, ATCC Accession Number CRL 9096) are among the host cells preferred for DHFR selection of a stably transfected cell line or DHFR-mediated gene amplification.

A cytochrome P450 may also be produced in insect cells, such cells include, without limitation, Spodoptera frugiperda (5S-9, SF-21, or Drosophila melanogaster Schneider (SL-2) cells. For P450 production, insect cells are typically infected with a baculovirus, for example, Autographa californica Multiple Nuclear Polyhedrosis Virus (AcMNPV) containing an expression cassette for such a protein, e.g., cytochrome P450, at a multiplicity of infection of 1 to 10. The infected cells are generally cultured in a standard insect cell culture medium for 24 to 48 hours prior to recovering the protein using standard molecular biology techniques. If desired, a P450 polypeptide may also be produced in insect cells directly transfected with a DNA construct containing an expression cassette encoding the P450.

Furthermore, any of the cytochrome P450s described herein may be produced in yeast, for example, Pichia pastoris. In order to produce the P450, yeast cells are transformed with an expression cassette containing, for example, a promoter such as the AOX1 or phosphoglycerate kinase gene promoter, the P450 gene to be expressed, and a terminator. Such an expression cassette may contain an origin of replication or it may be integrated into the yeast genomic DNA. The expression cassette is generally introduced by lithium acetate transformation or by the use of spheroplasts. In order to select for successfully transformed cells, the yeast are plated, for example, on minimal media which only allows yeast carrying the introduced expression cassette to grow.

In addition, expression of recombinant proteins in yeast using a Hansenula polymorpha expression system is described in U.S. Pat. Nos. 5,741,674 and 5,672,487. A P450 may also be produced by a stably-transfected plant cell line or by a transgenic plant. Such genetically-engineered plants are useful for a variety of industrial and agricultural applications as discussed below. Importantly, this invention is applicable to gymnosperms and angiosperms, and will be readily applicable to any new or improved transformation or regeneration method.

A number of vectors suitable for stable transfection of plant cells or for the establishment of transgenic plants are available to the public; such vectors are described in Poutewels et al. (supra), Weissbach and Weissbach (supra), and Gelvin et al. (supra). Methods for constructing such cell lines are described in, e.g., Weissbach and Weissbach (supra), and Gelvin et al. (supra). Typically, plant expression vectors include (1) a cloned P450 gene under the transcriptional control of 5' and 3' regulatory sequences and (2) a dominant selectable marker. Such plant expression vectors may also contain, if desired, a promoter regulatory region (for example, one conferring inducible or constitutive expression, or environmentally- or developmentally-regulated, or pathogen- or wound-inducible, or cell- or tissue-specific expression), a transcription initiation start site, a ribosome binding site, an RNA processing signal, a transcription termination site, and/or a polyadenylation signal.

The P450 DNA sequence of the invention may, if desired, be combined with other DNA sequences in a variety of ways. The P450 DNA sequence of the invention may be employed with all or part of the gene sequences normally associated with a P450. In its component parts, a DNA sequence encoding a P450 is combined in a DNA construct having a transcription initiation control region capable of promoting transcription and translation in a host cell.

In general, the constructs will involve regulatory regions functional in plants which provide for production of a P450 as discussed herein. The open reading frame coding for the P450, or a functional fragment thereof, will be joined at its 5' end to a transcription initiation regulatory region such as the sequence naturally found in the 5' upstream region of a P450 structural gene, for example, a CYP71D20 (SEQ ID NO: 2) or CYP71D21 (SEQ ID NO: 4) gene. Numerous other transcription initiation regions are available which provide for constitutive or inducible regulation.

For applications when developmental, cell, tissue, hormonal, environmental, or pathogen-inducible expression are desired, appropriate 5' upstream non-coding regions are
obtained from other genes; for example, from genes regulated during seed development, embryo development, leaf development, or in response to a pathogen.

Regulatory transcript termination regions may also be provided in DNA constructs of this invention as well. Transcript termination regions may be provided by the DNA sequence encoding a P450 or any convenient transcription termination region derived from a different gene source. The transcript termination region will contain preferably at least 1-3 kb of sequence 3' to the structural gene from which the termination region is derived.

An example of a useful plant promoter according to the invention is a cauliflower mosaic virus (CaMV) promoter, such as, a cauliflower mosaic virus (CaMV) promoter. These promoters confer high levels of expression in most plant tissues, and the activity of these promoters is not dependent on virally encoded proteins. CaMV is a source for both the 3SS and 19SS promoters. In most tissues of transgenic plants, the CaMV 3SS promoter is a strong promoter (see, e.g., Odell et al., Nature 313:810, 1985; the CaMV promoter is also highly active in monocots (see, e.g., Dekeserdt et al., Plant Cell 2:591, 1990; Teraoka and Shimamoto, Mol. Gen. Genet. 220:389, 1990). Moreover, activity of this promoter can be further increased (i.e., between 2-10 fold) by duplication of the CaMV 3SS promoter (see e.g., Kay et al., Science 236:1299, 1987; Ow et al., Proc. Natl. Acad. Sci. U.S.A. 84:4870, 1987; and Fang et al., Plant Cell 1:141, 1989). Other useful plant promoters include, without limitation, the nopaline synthase promoter (An et al., Plant Physiol. 88:547, 1988) and the octopine synthase promoter (Fromm et al., Plant Cell 1:977, 1989).

For certain applications, it may be desirable to produce the P450 gene product in an appropriate tissue, at an appropriate level, or at an appropriate developmental time. For this purpose, there is an assortment of gene promoters, each with its own distinct characteristics embodied in its regulatory sequences, which have been shown to be regulated in response to the environment, hormones, and/or developmental cues. These include gene promoters that are responsible for heat-regulated gene expression (see, e.g., Callis et al., Plant Physiol. 88:965, 1988; Takahashi and Komeda, Mol. Gen. Genet. 219:365, 1989; and Takahashi et al., Plant J. 2:751, 1992); light-regulated gene expression (e.g., the pea rbcS-3A described by Kuhlmeier et al. (Plant Cell 1:471, 1989); the maize rbcS promoter described by Schaffner and Sheen (Plant Cell 3:997, 1991); or the chlorophyll a/b-binding protein gene found in pea described by Simpson et al. (EMBO J. 4:2723, 1985)); hormone-regulated gene expression (for example, the abscisic acid (ABA) responsive sequences from the I'm gene of wheat described by Marcotte et al. (Plant Cell 1:969, 1989); the ABA-inducible HVA1 and HVA22, and the nd2A promoters described for barley and Arabidopsis by Straub et al. (Plant Cell 6:617, 1994), Shen et al. (Plant Cell 7:295, 1994)); and wound-induced gene expression (for example, of wun described by Siebertz et al. (Plant Cell 1:961, 1989); or organ-specific gene expression (for example, of the tuber-specific storage protein gene described by Rosthal et al. (EMBO J. 6:1155, 1987); the 23-kDa zein gene from maize described by Schermthner et al. (EMBO J. 7:1249, 1988); or the French bean beta-phaseolin gene described by Busto et al. (Plant Cell 1:839, 1989); and pathogen-inducible gene expression described by Chappell et al. in U.S. Ser. Nos. 08/471,983; 08/443,639; and 08/577,483; hereby incorporated by reference.

Plant expression vectors may also optionally include RNA processing signals, for example, introns, which have been shown to be important for efficient RNA synthesis and accumulation (Callis et al., Genes and Dev. 1:1183, 1987). The location of the RNA splice sequences can dramatically influence the level of transgene expression in plants. In view of this fact, an intron may be positioned upstream or downstream of a P450-encoding sequence in the transgene to modulate levels of gene expression.

In addition to the aforementioned 5' regulatory control sequences, the expression vectors may also include regulatory control regions which are generally present in the 3' regions of plant genes (Thornburg et al., Proc. Natl. Acad. Sci. U.S.A. 84:744, 1987; An et al., Plant Cell 1:115, 1989). For example, the 3'-terminal region may be included in the expression vector to increase stability of the mRNA. One such terminal region may be derived from the PH-II terminator region of potato. In addition, other commonly used terminators are derived from the octopine or nopaline synthase signals.

The plant expression vector also typically contains a dominant selectable marker gene used to identify cells that have become transformed. Useful selectable genes for plant systems include genes encoding antibiotic resistance genes, for example, those conferring resistance to hygromycin, kanamycin, bleomycin, G418, streptomycin, or spectinomycin. Genes required for photosynthesis may also be used as selectable markers in photosynthetically deficient strains. Alternatively, the green-fluorescent protein from the jellyfish Aequorea victoria may be used as a selectable marker (Sheen et al., Plant J. 8:777, 1995; Chiu et al., Current Biology 6:325, 1996). Finally, genes encoding herbicide resistance may be used as selectable markers; useful herbicide resistance genes include the bar gene encoding the enzyme phosphinothricin acetyltransferase and conferring resistance to the broad-spectrum herbicide BASTA (Hoechst AG, Frankfurt, Germany).

Efficient use of selectable markers is facilitated by a determination of the susceptibility of a plant cell to a particular selectable agent and a determination of the concentration of this agent which effectively kills most, if not all, of the transformed cells. Some useful concentrations of antibiotics for tobacco transformation include, e.g., 75-100 μg/ml (kanamycin), 20-50 μg/ml (hygromycin), or 5-10 μg/ml (bleomycin). A useful strategy for selection of transformants for herbicide resistance is described, e.g., by Vasil et al., supra.

It should be readily apparent to one skilled in the art of molecular biology, specifically in the field of plant molecular biology, that the level of gene expression is dependent, not only on the combination of promoters, RNA processing signals, and terminator elements, but also on how these elements are used to increase the levels of selectable marker gene expression.

Plant Transformation

Up on construction of the plant expression vector, several standard methods are available for introduction of the vector into a plant host, thereby generating a transgenic plant. These methods include (1) Agrobacterium-mediated transformation (A. tumefaciens or A. rhizogenes) (see, e.g., Lichtenstein and Fuller, In: Genetic Engineering, vol. 6, PWJ Rigby, ed. London, Academic Press, 1987; and Lichtenstein, C. P., and Draper, J., In: DNA Cloning, Vol II, D. M. Glover, ed, Oxford, IRI Press, 1985); (2) the particle delivery system (see, e.g., Gordon-Kamm et al., Plant Cell 2:603, 1990; or BioRad Technical Bulletin 1687, supra); (3) microinjection protocols (see, e.g., Green et al., supra); (4) polyethylene glycol (PEG) procedures (see, e.g., Draper et al., Plant Cell Physiol. 23:451, 1982; or e.g., Zhang and Wu, Theor. Appl. Genet. 76:835, 1988); (5) liposome-mediated DNA uptake (see, e.g., Freeman et al., Plant Cell Physiol. 25:1353, 1984); (6) electroporation protocols (see, e.g., Gelvin et al., supra; Dekeserdt et al., supra; Fromm et al., Nature 319:791, 1986; Sheen,
Plant Cell 2:1027, 1990; or Kang and Sheen, Plant Cell 6:1665, 1994); and (7) the vortexing method (see, e.g., 
Kindle, supra). The method of transformation is not critical to the present invention. Any method which provides for efficient 
transformation may be employed. As newer methods are available to transform crops or other host cells, they may be directly 
applied.

The following is an example outlining one particular technique, an Agrobacterium-mediated plant transformation. By 
this technique, the general process for manipulating genes to be transferred into the genome of plant cells is carried out in 
two phases. First, cloning and DNA modification steps are carried out in E. coli, and the plasmid containing the gene 
construct of interest is transferred by conjugation or electroporation into Agrobacterium. Second, the resulting Agro 
bacterium strain is used to transform plant cells. Thus, for the 
generalized plant expression vector, the plasmid contains an 
origin of replication that allows it to replicate in Agrobacte 
rium and a high copy number of origin of replication functional in E. coli. This permits lucifer production and testing of trans 
genesis in E. coli prior to transfer to Agrobacterium for subse 
quent introduction into plants. Resistance genes can be car 
rried on the vector, one for selection in bacteria, for example, streptomycin, and another that will function in plants, for 
example, a gene encoding kanamycin resistance or herbicide 
resistance. Also present on the vector are restriction endonu 
clase sites for the addition of one or more transgenes and 
directional T-DNA border sequences which, when recog 
nized by the transfer functions of Agrobacterium, delimit the 
DNA region that will be transferred to the plant.

In another example, plant cells may be transformed by 
shooting into the cell tungsten microprojectiles on which 
cloned DNA is precipitated. In the Biologic Apparatus (Bio 
Rad) used for the shooting, a gunpowder charge (22 caliber 
Power Piston Tool Charge) or an air-driven blast drives a 
plastic macroprojectile through a gun barrel. An aliquot of 
a suspension of tungsten particles on which macroprojectile 
has been precipitated is placed on the front of the plastic macroproject 
ile. The latter is fired at an acrylic stopping plate that has a 
hole through it that is too small for the macroprojectile to pass 
through. As a result, the plastic macroprojectile smashes 
against the stopping plate, and the tungsten microprojectiles 
continue toward their target through the hole in the plate. 
For the present invention, the target can be any plant cell, tissue, 
seed, or embryo. The DNA introduced into the cell on the 

microprojectiles becomes integrated into either the nucleus or 
the chloroplast.

In general, transfer and expression of transgenes in plant 
cells are now routine practices to those skilled in the art, 
and have become major tools to carry out gene expression studies in plants and to produce improved plant varieties of agruc 
tural or commercial interest.

Transgenic Plant Regeneration

Plants cells transformed with plant expression vectors can be 
regenerated, for example, from single cells, callus tissue, 
or leaf discs according to standard plant tissue culture tech 
niques. It is well known in the art that various cells, tissues, 
and organs from almost any plant can be successfully cultured to 
regenerate an entire plant; such techniques are described, e.g., in 
Vasil (supra), Green et al. (supra), Weissbach and 
Weissbach (supra) and Gelvin et al. (supra).

In one particular example, a cloned P450, under the control 
of the EAS4 promoter and the nopaline synthase terminator 
and carrying a selectable marker (for example, kanamycin 
resistance), is transformed into Agrobacterium. Transfor 
mation of leaf discs (for example, of tobacco leaf discs), with 
vector-containing Agrobacterium is carried out as described 
by Horsch et al. (Science 227:1229, 1985). Putative transformants 
are selected after a few weeks (for example, 3 to 5 
weeks) on plant tissue culture media containing kanamycin 
(e.g., 100µg/ml). Kanamycin-resistant shoots are then placed on 
plant tissue culture media without hormones for root initia 
tion. Kanamycin-resistant plants are then selected for 
green house growth. If desired, seeds from self-fertilized 
transgenic plants can then be sown in soil-less medium and 
grown in a greenhouse. Kanamycin-resistant progeny are 
selected by sowing surface sterilized seeds on hormone-free 
kanamycin-containing media.

Analysis for the integration of the transgene is accom 
plished by standard techniques (see, for example, Ausubel et 
(al. supra); Gelvin et al. (supra)).

Transgenic plants expressing the selectable marker are 
then screened for transmission of the transgene DNA by 
standard immunoblot and DNA detection techniques. Each 
positive transgenic plant and its transgenic progeny is unique 
in comparison to other transgenic plants established with the 
same transgene. Integration of the transgene DNA into the 
plant genomic DNA is in most cases random, and the site of 
integration can profoundly affect the levels and the tissue and 
developmental patterns of transgene expression. Consequent 
ly, a number of transgenic lines are usually screened for 
each transgene to identify and select plants with the most 
appropriate expression profiles.

Transgenic lines are generally evaluated for levels of trans 
gene expression. Expression at the RNA level is determined 
initially to identify and quantitate expression-positive plants. 
Standard techniques for RNA analysis are employed and 
include PCR amplification assays using oligonucleotide primers designed to amplify only transgene RNA templates and 
solution hybridization assays using transgene-specific probes (see, e.g., Ausubel et al. (supra)). The RNA-positive 
plants are then analyzed for protein expression by Western 
immunoblot analysis using specific antibodies to the P450 
(see, e.g., Ausubel et al., supra). In addition, in situ hybrid 
ization and immunocytochemistry according to standard pro 
tocols can be done using transgene-specific nucleotide probes 
and antibodies, respectively, to localize sites of expression 
within transgenic tissue.

Once the recombinant P450 is expressed in any cell in a 
transgenic plant (for example, as described above), it may 
be isolated, e.g., using affinity chromatography. In one example, 
an anti-P450 antibody (e.g., produced as described in 
Ausubel et al., supra, or by any standard technique) may be attached to 
a column and used to isolate the polypeptide. Lysis and fraction 
ation of P450-producing cells prior to affinity chromatography 
may be performed by standard methods (see, e.g., 
Ausubel et al., supra). Once isolated, the recombinant protein 
can, if desired, be further purified, for example, by high 
performance liquid chromatography (see, e.g., Fisher, Labo 
atory Techniques in Biochemistry and Molecular Biology, 

These general techniques of polypeptide expression and 
purification can also be used to produce and isolate useful 
P450 fragments or analogs.

Use

The aforementioned cytochrome P450 polypeptides of the 
invention are useful in the biosynthesis of hormones, lipids, 
and secondary metabolites, and may also help plants tolerate 
potentially harmful exogenous chemicals such as herbicides, 
pesticides, and pollutants. In addition, such cytochrome P450 
polypeptides are useful in the chemical defense of plants 
against insects, as well as against bacterial, viral, and fungal 
infection.
Engineering Plant Disease Resistance

Plasmid constructs designed for the expression of a P450 gene product are useful, for example, for activating plant defense pathways that confer anti-pathogen properties to a transgenic plant, for example, the production of phytoalexins. P450 genes that are isolated from a host plant (e.g., Nicotiana) may be engineered for expression in the same plant, a closely related species, or a distantly related plant species. For example, a P450 gene may be engineered for constitutive low-level expression and then transformed into a Nicotiana host plant. Alternatively, the P450 gene may be engineered for expression in other solanaceous plants, including, but not limited to, potato and tomato. To achieve pathogen resistance, it is important to express a P450 protein at an effective level. Evaluation of the level of pathogen protection conferred to a plant by ectopic expression of the P450 gene is determined according to conventional methods and assays.

Industrial Applications

The invention also includes engineering host cells to include novel isoprenoid metabolic pathways useful in the production of new isoprenoid compounds. By introducing genes encoding an isoprenoid synthase (as disclosed in U.S. Pat. No. 5,824,774 and WO 00/17327) and a cytochrome P450, an acetyltransferase, a methyl transferase, a fatty acyltransferase, or a combination thereof, various isoprenoid reaction products may be modified, controlled, or manipulated, resulting in enhancement of production of numerous isoprenoid reaction products, for example, the production of novel monoterpenses, diterpenes, and sesquiterpenes. Such compounds are useful as phytoalexins, insecticides, perfumes, and pharmaceuticals such as anti-bacterial and fungal agents.

In one working example, an isoprenoid synthase or a chimeric isoprenoid synthase (as disclosed in U.S. Pat. No. 5,824,774 and WO 00/17327) and a P450 gene are introduced into yeast, for example, using any of the procedures described herein. If desired, such cells may also express, either independently or in combination, an acetyltransferase (see, for example, Walker et al., Proc. Natl. Acad. Sci. U.S.A. 18:583-587, 2000), a methyltransferase gene (see, for example, Diener et al., Plant Cell 12:853-870, 2000), or a fatty acyltransferase gene, as well as a cytochrome reductase. Cells are then cultured under standard conditions and the production of isoprenoid compounds is assayed according to methods known in the art. Isoprenoid compounds are further purified according to methods well known in the art. Cells expressing novel isoprenoid compounds are taken as useful in the invention.

Such methods provide a unique approach for producing novel isoprenoid starting materials and end products. Either prokaryotic or eukaryotic cells transformed with any of the aforementioned enzymes (or combinations thereof) may be used. Moreover, isoprenoid compounds may be produced in any number of ways known in the art including an in vitro combination of purified enzymes with an appropriate substrate or direct fermentation using a host cell which expresses any combination of the aforementioned enzymes and the appropriate substrates sufficient to drive production of isoprenoid compounds.

The invention is also useful for the production of insect attractants and deterrents, which may either deter insect pests or attract insect predators. In addition, the invention is also useful for generating novel flavorings and perfumes.

Other Embodiments

From the foregoing description, one skilled in the art can easily ascertain the essential characteristics of this invention, and can make various changes and modifications of the invention to adapt it to various usages and conditions. Thus, other embodiments are also within the claims.

All publications and patents mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent was specifically and individually indicated to be incorporated by reference.
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Ile Trp Phe Ala Ser Ser Met Thr Cys Arg Ser Ala Phe Gly Gln Val 180 185 190
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Lys Tyr Leu Lys Leu Val Ile Lys Glu Thr Leu Arg Leu Aam Pro Pro 325 330 335
Ser Pro Leu Leu Val Pro Arg Gly Cys Arg Glu Asp Thr Asp Ile Aam 340 345 350
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Leu Gly Arg Asp Pro Lys Tyr Trp Asp Asp Ala Glu Ser Phe Lys Pro 370 375 380
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Phe Leu Pro Phe Gly Gly Gly Arg Ile Cys Pro Gly Met Ser Phe 405 410 415
Gly Leu Ala Aam Leu Tyr Leu Pro Leu Ala Glu Leu Leu Tyr His Phe 420 425 430
Asp Trp Lys Leu Pro Thr Gly Ile Met Pro Arg Asp Leu Asp Leu Thr 435 440 445
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20     25         30

Pro Pro Gly Pro Trp Lys Ile Pro Ile Leu Gly Ser Met Leu His Met
35     40         45

Leu Gly Gly Pro His Ile Leu Arg Asp Leu Ala Lys Tyr
50     55         60

Gly Pro Ile Met His Leu Gln Phe Gly Ile Ser Ala Val Val Val
65     70         75         80

Thr Ser Arg Glu Met Ala Lys Gly Val Leu Lys Thr His Asp Val Val
85     90         95

Phe Ala Ser Arg Pro Lys Ile Val Ala Met Asp Ile Ile Cys Tyr Asn
Gln Ser Asp Ile Ala Phe Ser Pro Tyr Gly Asp His Trp Arg Gln Met 115
Arg Lys Ile Cys Val Met Glu Leu Leu Asn Ala Lys Asn Val Arg Ser 130
Phe Ser Ser Ile Arg Arg Asp Glu Val Val Arg Leu Ile Asp Ser Ile 145
Arg Ser Asp Ser Ser Gly Glu Leu Val Asn Phe Thr Gln Arg Ile 160
Ile Trp Phe Ala Ser Ser Met Thr Cys Arg Ser Ala Phe Gly Gln Val 175
Leu Lys Gly Gln Asp Val Phe Ala Lys Lys Ile Arg Glu Val Ile Gly 190
Leu Ala Glu Gly Phe Asp Val Ala Asp Ile Phe Pro Ser Tyr Lys Phe 205
Leu His Val Leu Ser Gly Met Lys Arg Lys Leu Leu Asn Ala His Leu 220
Lys Val Asp Ala Ile Val Glu Asp Val Ile Asn Glu His Lys Lys Asn 235
Leu Ala Thr Gly Lys Thr Asn Gly Ala Leu Gly Gly Asp Met Phe Ala 250
Ala Gly Thr Glu Thr Ser Ser Thr Thr Thr Val Trp Ala Met Ala Glu 265
Met Met Lys Asn Pro Asn Val Phe Asn Lys Ala Gln Ala Glu Val Arg 280
Glu Thr Phe Lys Asp Lys Val Thr Phe Asp Glu Ile Asp Ala Glu Glu 295
Leu Glu Tyr Leu Lys Leu Val Ile Lys Glu Thr Leu Arg Leu His Pro 310
Pro Ser Pro Leu Leu Val Val Arg Asn Leu Gly Asp Thr Asp Ile 325
Leu Thr Tyr Thr Ser Ala Ile Pro Leu Lys Thr Val Met Val Asn Val Trp 340
Glu Tyr Ala Gly Arg Asp Pro Lys Tyr Trp Asp Asp Ala Glu Ser Phe Lys 355
Pro Glu Arg Phe Glu Gln Cys Ser Val Asp Phe Phe Asp Gly Asn Asn Phe 370
Glu Lys Tyr Leu Ala Gly Lys Phe Leu Phe Gly Gly Arg Asp Ile Cys Pro Gly Met Ser 385
Phe Lys Leu Ala Asn Leu Tyr Leu Pro Leu Ala Glu Leu Leu Tyr His 400
Phe Asp Trp Lys Leu Pro Ser Gly Met Met Pro Gly Asp Leu Asp Leu 415
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Met Ala Thr Pro Tyr Gln Pro Ser Arg Glu 445

<210> SEQ ID NO 4
<211> LENGTH: 1614
<212> TYPE: DNA
<213> ORGANISM: Nicotiana tabacum
<400> SEQUENCE: 4

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<400> SEQUENCE: 5

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35 40 45
Pro Leu Ile Ile Asn Phe Leu Tyr Val Lys Pro Glu Asn Asn Leu Pro
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Pro Gly Pro Thr Ala Val Pro Ile Phe Gly Asn Thr Leu Glu Val Gly
65 70 75 80
Asn Asp Leu Asn His Glu Leu Ala Thr Met Ser Glu Thr Tyr Glu
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<211> LENGTH: 1745
<212> TYPE: DNA
<213> ORGANISM: Nicotiana tabacum

<400> SEQUENCE: 6

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<210> SEQ ID NO 7
<211> LENGTH: 534
<212> TYPE: PRT
<213> ORGANISM: Nicotiana tabacum

<400> SEQUENCE: 7

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20  25  30
Phe Pro Leu Glu Tyr Ile Ser Leu Ile Val Leu Leu Leu Pro Leu Ile
35  40  45
Ile Asn Phe Leu Cys Val Lys Pro Glu Asn Asn Leu Pro Pro Gly Pro
50  55  60
Thr Ala Val Pro Ile Phe Gly Asn Trp Leu Glu Val Gly Asn Asp Leu
65  70  75  80
Asn His Glu Leu Leu Ala Thr Met Ser Glu Thr Tyr Gly Pro Ile Phe
85  90  95
Leu Leu Lys Leu Gly Ser Lys Asn Leu Ala Val Val Ser Asn Pro Glu
100 105 110
Leu Ala Asn Glu Val Leu His Thr Glu Gly Val Glu Phe Gly Ser Arg
115 120 125
Pro Arg Asn Val Phe Asp Ile Phe Thr Gly Asn Gly Glu Asp Met
130 135 140
Val Phe Thr Ile Tyr Gly Asp His Thr Arg Lys Met Arg Arg Ile Met
145 150 155 160
Thr Leu Pro Phe Phe Thr Asn Lys Val Val His Glu Tyr Ser Asp Met
165 170 175
Trp Glu Asn Glu Met Asp Leu Val Val Asp Asp Leu Lys Lys Asn Glu
180 185 190
Lys Val Lys Tyr Asp Gly Ile Val Ile Arg Lys Arg Leu Glu Glu Leu Met
195 200 205
Leu Tyr Asn Ile Met Tyr Arg Met Met Phe Asp Ala Lys Phe Glu Ser
210 215 220
Gln Asp Asp Pro Leu Phe Ile Glu Ala Thr Lys Phe Asn Ser Glu Arg
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Ser Arg Leu Ala Glu Ser Phe Asp Tyr Asn Tyr Gly Asp Pro Ile Pro
245 250 255
Leu Leu Arg Pro Phe Leu Lys Gly Tyr Leu Asn Lys Ala Lys Asp Leu
260 265 270
Gln Thr Arg Arg Leu Ala Phe Asp Asn Tyr Phe Val Gly Lys Arg
275 280 285
Arg Lys Ile Met Gly Glu Asn Gly Glu Lys His Lys Ile Cys Cys Ala
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Ile Asp His Ile Ile Asp Ala Glu Met Lys Gly Glu Ile Ser Glu Gln
305 310 315 320
Asn Val Leu Tyr Ile Val Glu Asn Ile Asn Val Ala Ala Ile Glu Thr
325 330 335
Thr Leu Trp Ser Met Glu Trp Ala Ile Ala Glu Leu Val Asn His Pro
340 345 350
Ile Val Glu Glu Gly Ile Arg Asp Glu Ile Ser Thr Val Leu Lys Gly
355 360 365
Lys Ser Val Lys Glu Ser Asn Leu His Glu Pro Tyr Leu Lea Ala
370 375 380
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Pro His Met Asn Leu Glu Glu Ala Lys Leu Gly Gly Tyr Thr Ile Pro
405 410 415
Lys Glu Thr Lys Val Val Asn Ala Trp Trp Leu Ala Asn Asn Pro
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Glu Asp Ser Ser Thr Glu Ala Ala Val Ala Gly Gly Lys Val Asp Phe
450 455 460
Arg Tyr Leu Pro Phe Gly Met Gly Arg Arg Ser Cys Pro Gly Ile Ile
465 470 475 480
Leu Ala Leu Pro Ile Leu Gly Leu Val Ile Ala Lys Leu Val Ser Arg
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Phe Glu Met Gln Ala Pro Pro Gly Val Gly Lys Val Asp Thr Ser Glu
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Lys Gly Gly Gln Phe Ser Leu His Ile Ala Lys His Ser Thr Val Val
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<210> SEQ ID NO 8
<211> LENGTH: 1693
<212> TYPE: DNA
<213> ORGANISM: Nicotiana tabacum

<400> SEQUENCE: 8

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<210> SEQ ID NO: 9
<211> LENGTH: 519
<212> TYPE: PRT
<213> ORGANISM: Nicotiana tabacum

<400> SEQUENCE: 9

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20  25  30
Leu Asn Pro Leu Pro Pro Gly Gly Trp Pro Val Ile Gly
35  40  45
His Leu Phe Tyr Phe Asn Asn Gly Asp Asp Arg His Phe Ser
50  55  60
Gln Lys Leu Gly Asp Leu Ala Asp Lys Tyr Gly Pro Val Phe Thr Phe
65  70  75  80
Arg Leu Gly Phe Arg Arg Phe Leu Ala Val Ser Ser Tyr Glu Ala Met
85  90  95
Lys Glu Cys Phe Ser Thr Asn Asp Ile His Phe Ala Asp Arg Pro Ala
100 105  110
Leu Leu Tyr Gly Glu Tyr Leu Cys Tyr Asn Asn Ala Met Leu Ala Val
115 120  125
Ala Lys Tyr Gly Pro Tyr Trp Lys Asn Arg Lys Leu Val Asn Gln
130 135 140
Glu Leu Leu Ser Val Ser Arg Leu Glu Lys Phe His Val Arg Phe
145 150  155 160
Ser Ile Val Glu Glu Asn Ile Lys Glu Leu Tyr Asn Cys Asp Ser Pro
165 170  175
Met Val Lys Ile Asn Leu Ser Asp Trp Ile Asp Lys Leu Thr Phe Asp
180 185  190
Ile Ile Leu Lys Met Val Val Gly Lys Thr Tyr Asn Asn Gly His Gly
195 200  205
Glu Ile Leu Lys Ala Ala Phe Gln Lys Phe Met Val Glu Ala Met Glu
210 215  220
Ile Glu Leu Tyr Asp Val Phe His Ile Pro Phe Phe Lys Trp Leu Asp
225 230  235  240
Leu Thr Gly Asn Ile Lys Ala Met Lys Glu Thr Phe Lys Asp Ile Asp
245 250  255
Asn Ile Ile Gln Gly Trp Leu Asp Glu His Ile Lys Arg Glu Thr
260 265  270
Lys Asp Val Gly Gly Glu Asn Glu Glu Asp Phe Ile Asp Val Leu Leu
275 280  285
Ser Lys Arg Ser Asn Glu His Leu Gly Asp Gly Tyr Ser His Asp Thr
290 295  300
Thr Ile Lys Ala Thr Val Phe Thr Leu Val Leu Asp Ala Thr Asp Thr
305 310  315  320
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Asn Val Met Met Lys Lys Ala Gln Glu Glu Met Asp Thr Ile Val Gly Arg
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SEQ ID NO: 10
LENGTH: 1578
TYPE: DNA
ORGANISM: Nicotiana tabacum

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Gly Gly Thr Glu Ser Ser Ala Val Thr Val Glu Trp Ala Ile Ser Glu
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325 330 335
Arg Val Ile Gly Gln Asn Arg Trp Val Gin Glu Lys Asp Ile Pro Asn
340 345 350
Leu Pro Tyr Ile Glu Ala Ile Val Lys Glu Thr Met Arg Leu His Pro
355 360 365
Val Ala Pro Met Leu Val Pro Arg Glu Cys Arg Glu Asp Cys Lys Val
370 375 380
Ala Gly Tyr Asp Val Lys Gly Thr Arg Val Leu Val Ser Val Trp
385 390 395 400
Thr Ile Gly Arg Asp Pro Thr Leu Trp Asp Glu Pro Ala Phe Lys
405 410 415
Pro Glu Arg Phe His Glu Lys Ser Ile Asp Val Lys Gly His Asp Phe
420 425 430
Glu Leu Leu Pro Phe Gly Ala Gly Arg Arg Met Cys Pro Gly Tyr Asn
435 440 445
Leu Gly Leu Lys Val Ile Gin Ala Ser Leu Ala Asn Leu Ile His Gly
450 455 460
Phe Asn Trp Ser Leu Pro Asp Asn Met Thr Pro Glu Asp Leu Asp Met
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Asp Glu Ile Phe Gly Leu Ser Thr Pro Lys Lys Phe Pro Leu Ala Thr
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Val Ile Glu Pro Arg Leu Ser Pro Lys Leu Tyr Ser Val
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<212> TYPE: DNA
<213> ORGANISM: Nicotiana tabacum

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ctgctaaaga agccagagat tttccaaagag gctacagaga aatgggatcg agtaatgggg

<210> SEQ ID NO 13
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<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURES:
<223> OTHER INFORMATION: Derived from Nicotiana tabacum p450 gene
<400> SEQUENCE: 13

ggccagagat tgtccttgga atgctatgtag gtttag

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<220> FEATURES:
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<400> SEQUENCE: 14

gtacaaagt gaggttgacga atg

<210> SEQ ID NO 15
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<220> FEATURES:
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ggtggtgtgat atgcatag

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<210> SEQ ID NO 17
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<223> OTHER INFORMATION: Derived from Nicotiana tabacum p450 gene
<400> SEQUENCE: 17

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<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
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<210> SEQ ID NO 21
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<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Derived from Nicotiana tabacum p450 gene

<400> SEQUENCE: 21
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<210> SEQ ID NO 22
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<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
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<210> SEQ ID NO 23
<211> LENGTH: 76
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
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<223> OTHER INFORMATION: Derived from Nicotiana tabacum p450 gene

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<210> SEQ ID NO 24
<211> LENGTH: 75
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
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OTHER INFORMATION: Derived from Nicotiana tabacum p450 gene

SEQ ID NO: 24
LENGTH: 33
TYPE: DNA
ORGANISM: Artificial sequence
FEATURE:
NAME/KEY: Variant
LOCATION: (7)...(7)
OTHER INFORMATION: Xaa = any amino acid

SEQ ID NO: 26
LENGTH: 7
TYPE: PRT
ORGANISM: Nicotiana tabacum p450 protein
FEATURE:
NAME/KEY: Variant
LOCATION: (7)...(7)
OTHER INFORMATION: Xaa = any amino acid

NAME/KEY: Variant
LOCATION: (8)...(8)
OTHER INFORMATION: Xaa = any amino acid

SEQ ID NO: 28
LENGTH: 11
TYPE: PRT
ORGANISM: Nicotiana tabacum p450 protein
FEATURE:
NAME/KEY: Variant
LOCATION: (4)...(4)
OTHER INFORMATION: Xaa = any amino acid

NAME/KEY: Variant
LOCATION: (8)...(8)
OTHER INFORMATION: Xaa = any amino acid

SEQ ID NO: 29
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TYPE: PRT
ORGANISM: Nicotiana tabacum p450 protein
FEATURE:
NAME/KEY: Variant
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<223> OTHER INFORMATION: Xaa = any amino acid

<400> SEQUENCE: 29

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<210> SEQ ID NO 30
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<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
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Thr Ile Cys Ala
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Thr Ile Thr Ala
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Thr Ile Met Gly
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Thr Tyr

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Ala Ala

<210> SEQ ID NO 35
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Cys Ile Gly Ser
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<210> SEQ ID NO 36
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<212> TYPE: PRT
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<223> OTHER INFORMATION: Derived from Nicotiana tabacum p450 protein

<400> SEQUENCE: 36
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1  5 10 15

Ala Ile Gly Gly
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<210> SEQ ID NO 37
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<212> TYPE: DNA
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<220> FEATURE:
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<400> SEQUENCE: 37
gtaataagac tcactatagg g

<210> SEQ ID NO 38
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Derived from T3 bacteriophage promoter

<400> SEQUENCE: 38
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<210> SEQ ID NO 39
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<212> TYPE: PRT
<213> ORGANISM: Mentha piperita

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Thr Ile Ser Leu Leu Ile Ile Lys Gln Trp Arg Lys Pro Lys Pro Gln
20 25 30

Glu Asn Leu Pro Pro Gly Pro Pro Lys Leu Pro Leu Ile Gly His Leu
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Val Leu Ser Ser Ala Glu Ala Ala Lys Gln Ala Met Lys Val Leu Asp
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Tyr Asp Lys Asp Ile Ile Phe Ser Pro Tyr Asn Asp His Trp Arg
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Leu Gly Leu Tyr Pro His Arg Tyr Leu Gln Ser Leu Ser Arg Arg Tyr 50 55 60
Gly Pro Leu Met Gln Leu His Phe Gly Ser Val Pro Val Leu Val Ala 65 70 75 80
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Phe Ser Asn Arg Pro Lys Met Ser Ile Ala Asn Arg Leu Phe Phe Asn 100 105 110
Asn Arg Asp Val Ala Phe Thr Glu Tyr Gln Val Tyr Trp Arg Gln Ile 115 120 125
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<210> SEQ ID NO 43
<211> LENGTH: 509
<212> TYPE: PRT
<213> ORGANISM: Arabidopsis thaliana
<400> SEQUENCE: 43

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Lys Asn Met Ser Glu Val Ser Thr Leu Pro Ser Val Pro Val Val Pro
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Gly Phe Pro Val Ile Gly Asn Leu Leu Glu Leu Lys Glu 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 Ser Thr Leu Arg Leu Leu Asn Ser Thr Glu Thr
85 90 95

Ala Lys Glu Ala Met Val Thr Arg Phe Ser Ser Thr Ser Thr Arg 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 Glu 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 Glu Glu Pro Val Asn Phe Arg Ala Ile Phe Glu His Glu
180 185 190

Leu Phe Gly Val Ala Leu Lys Glu 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
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Arg Asp Phe Phe Pro Tyr Leu Lys Trp Ile Pro Asn Lys Ser Phe Glu
245 250 255

Ala Arg Ile Glu Glu Lys His Lys Arg Arg Leu Ala Val Met Asn Ala
260 265 270

Leu Ile Glu Asp Arg Leu Lys Glu Asn Gly Ser Glu Ser Ser 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 Glu Asp Arg Leu Cys Lys Glu Ile Glu Asn Val Cys Gly Gly
340 345 350

Glu Lys Phe Lys Glu Glu Leu Ser Glu Ser Glu Val 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 Glu Ile Gly Gly Tyr Hist His Val Pro
395 395 400

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
The invention claimed is:

1. A method for producing an isoprenoid compound, comprising:
   a) providing a host cell that expresses heterologous nucleic acid encoding a first recombinant protein comprising an isoprenoid synthase; and a second recombinant protein comprising a cytochrome P450 polypeptide, wherein: the isoprenoid synthase catalyzes production of an isoprenoid compound that is a diterpene or sesquiterpene; and the cytochrome P450 polypeptide catalyzes hydroxylation, oxidation, demethylation or methylation of the diterpene or sesquiterpene produced by the synthase to produce a diterpene or a sesquiterpene compound not normally produced by the host cell nor whose production is catalyzed by the synthase;
   b) culturing the host cell under conditions suitable for expressing the first recombinant protein comprising an isoprenoid synthase and the second recombinant protein comprising the cytochrome P450 polypeptide and under conditions for producing the diterpene or a sesquiterpene isoprenoid compound not normally produced by the host cell, wherein the first and second recombinant protein together catalyze the formation of a diterpene or sesquiterpene isoprenoid compound; and
   c) recovering the diterpene or sesquiterpene isoprenoid compound not normally produced by the host cell.

2. The method of claim 1, wherein the cytochrome P450 polypeptide is a CYP71, CYP73, CYP82 or CYP92 family cytochrome P450.

3. The method of claim 2, wherein each of the CYP71, CYP73, CYP82 and CYP92 family cytochrome P450 polypeptides is encoded by nucleic acid that can be amplified with degenerate primers based on one of SEQ ID NOS: 26-29.

4. The method of claim 1, wherein the cytochrome P450 polypeptide has dual hydroxylase activity.

5. The method of claim 3, wherein the CYP71, CYP73, CYP82 and CYP92 family cytochrome P450 polypeptide has dual hydroxylase activity.

6. A method for producing an altered isoprenoid compound, comprising:
   a) contacting the isoprenoid compound with an isolated cytochrome P450 polypeptide, wherein the P450 polypeptide has dual hydroxylase activity and catalyzes the dual hydroxylation of the isoprenoid compound to produce the altered isoprenoid compound wherein the cytochrome P450 polypeptide is a CYP71, CYP73, CYP82 or CYP92 family cytochrome P450 polypeptide; and
   b) recovering the altered isoprenoid compound.

7. The method of claim 6, wherein the isoprenoid compound is a sesquiterpene.

8. The method of claim 6, wherein the P450 polypeptide is a CYP71, CYP73, CYP82 or CYP92 family cytochrome P450 polypeptide encoded by nucleic acid that can be amplified with degenerate primers based on one of SEQ ID NOS: 26-29.

9. A host cell, comprising nucleic acid encoding a first recombinant protein comprising an isoprenoid synthase, and nucleic acid encoding a second recombinant protein comprising a cytochrome P450 polypeptide, wherein:
   the first and second recombinant proteins together catalyze the formation of an isoprenoid compound not normally produced by the host cell nor whose production is catalyzed by the isoprenoid synthase; and
   the first recombinant protein and the nucleic acid encoding the second recombinant protein are heterologous to the host cell;
   the P450 polypeptide has dual hydroxylation activity and catalyzes dual hydroxylation of the isoprenoid whose production is catalyzed by the synthase; and
   the second recombinant protein comprises a CYP71, CYP73, CYP82 or CYP92 family cytochrome P450 polypeptide.

10. The host cell of claim 9, wherein the isoprenoid is a sesquiterpene.

11. The host cell of claim 9, wherein the P450 polypeptide is encoded by nucleic acid that can be amplified with degenerate primers based on one of SEQ ID NOS: 26-29.

12. A host cell, comprising nucleic acid encoding a first recombinant protein comprising a diterpene or sesquiterpene isoprenoid synthase, and nucleic acid encoding a second recombinant protein comprising a cytochrome P450 polypeptide, wherein:
   the nucleic acid encoding the first recombinant protein, and the nucleic acid encoding the second recombinant protein are heterologous to the host cell;
   the synthase catalyzes production of a diterpene or sesquiterpene compound;
   the P450 polypeptide catalyzes the dual hydroxylation, oxidation, demethylation or methylation of the diterpene or sesquiterpene whose production is catalyzed by the synthase;
   the resulting diterpene or sesquiterpene compound is not normally produced by the host cell nor by the synthase in the absence of the P450 enzyme.

13. The host cell of claim 12, wherein the P450 polypeptide is a CYP71, CYP73, CYP82 and CYP92 family cytochrome P450 polypeptide encoded by nucleic acid that can be amplified with degenerate primers based on one of SEQ ID NOS: 26-29.

14. The host cell of claim 12, wherein the P450 polypeptide comprises a CYP71, CYP73, CYP82 or CYP92 family cytochrome P450 polypeptide.
15. The method of claim 1, wherein:
the isoprenoid compound is a sesquiterpene; and
the isoprenoid synthase is a sesquiterpene synthase.

16. The method of claim 10, wherein the P450 polypeptide
is a CYP71, CYP73, CYP82 or CYP92 family cytochrome
P450 polypeptide is encoded by nucleic acid that can be
amplified with degenerate primers based on one of SEQ ID
NOS: 26-29.

* * * * *
It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

IN THE SPECIFICATION:

at column 1, lines 55-56, please replace “mediated by an elicitor-inducible cytochrome P450 ¶(FIG. 1).” with --mediated by an elicitor-inducible cytochrome P450 (FIG. 1).--;

at column 2, lines 16-17, please replace “tolochene to 1-deoxycapsidiol was dependent on both ¶NADPH and O₂;” with --tolochene to 1-deoxycapsidiol was dependent on both NADPH and O₂--;

at column 2, line 26, please replace “ofcapsidiol” with --of capsidiol--;

at column 6, lines 6-7, please replace “11-16 of Agrios, ¶Plant Pathology” with --11-16 of Agrios, Plant Pathology--;

at column 7, line 53, please replace “WAT1 1” with --WAT11--;

at column 8, line 59, please replace “SEAH” with --5EAH--;

at column 8, line 63, please replace “SEAH” with --5EAH--;

at column 13, line 31, please replace “258 by” with --258 bp--;

at column 14, lines 22-24, please replace “5'-GCCATTATCGCGCAATACTAATC TTCAAACTCCGCAGTAAAAATTTCAAGCTCCCACTGGGTCAACAGCGATCT-3'” with --5'-GCCATTATCGCGCAATACTAATC TTCAAAACTCCGCAGTAAAAATTTCAAGCTCCCAACAGCGATCT-3'--;

Signed and Sealed this Twenty-fifth Day of February, 2014

Michelle K. Lee
Deputy Director of the United States Patent and Trademark Office
CERTIFICATE OF CORRECTION (continued)

U.S. Pat. No. 8,445,231 B2

at column 14, lines 25-27, please replace “5'-GGGGGATCCATGGACCTCCTCTCCTC
ATAGAAAAAACCCCTCGTCGCTTATTCGAGCCATTATCGGCGCAATTACTA-3’” with --5'-GGGGGATCCATGGACCTCCTCCTCCTCCTCCTATAGAAAAAACCCCTCGTCG
CTTATTCGAGCCATTATCGGCGCAATTACTA-3’--;

at column 15, line 67, please replace “100 nl” with --100 µl--;

at column 16, line 10, please replace “50 .tM” with --50 µM--;

at column 18, line 32, please replace “P.sub.L” with --P1--; and

at column 18, line 33, please replace “Simatke” with --Shimatake--.

IN THE CLAIMS:

Column 73, line 42 to line 44 should read:

2. The method of claim 1, wherein the cytochrome P450 polypeptide is a CYP71, CYP73,
CYP82 or CYP92 family cytochrome P450.

Column 75, line 4 to line 8, please delete duplicate Claim 16.