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Cytochrome P450s and Uses Thereof

Joe Chappell

University of Kentucky, chappell@uky.edu

Lyle F. Ralston

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(12) **United States Patent**
Chappell et al.(10) **Patent No.:** **US 8,445,231 B2**
(45) **Date of Patent:** **May 21, 2013**(54) **CYTOCHROME P450S AND USES THEREOF**(75) Inventors: **Joseph Chappell**, Lexington, KY (US);
Lyle F. Ralston, Chesterfield, MO (US)(73) Assignee: **University of Kentucky Research**
Foundation, Lexington, KY (US)(*) Notice: Subject to any disclaimer, the term of this
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U.S.C. 154(b) by 0 days.(21) Appl. No.: **13/199,349**(22) Filed: **Aug. 26, 2011**(65) **Prior Publication Data**

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C12N 5/00 (2006.01)
C12N 5/07 (2010.01)(52) **U.S. Cl.**USPC **435/69.1**; 435/468; 435/348; 435/252.1;
435/252.2; 435/155; 435/419(58) **Field of Classification Search**

None

See application file for complete search history.

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(Continued)

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(74) Attorney, Agent, or Firm — McKenna Long & Aldridge
LLP; Stephanie Seidman(57) **ABSTRACT**The invention features isolated cytochrome P450 polypep-
tides and nucleic acid molecules, as well as expression vec-
tors 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 com-
pounds.**16 Claims, 11 Drawing Sheets**

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FIG. 1

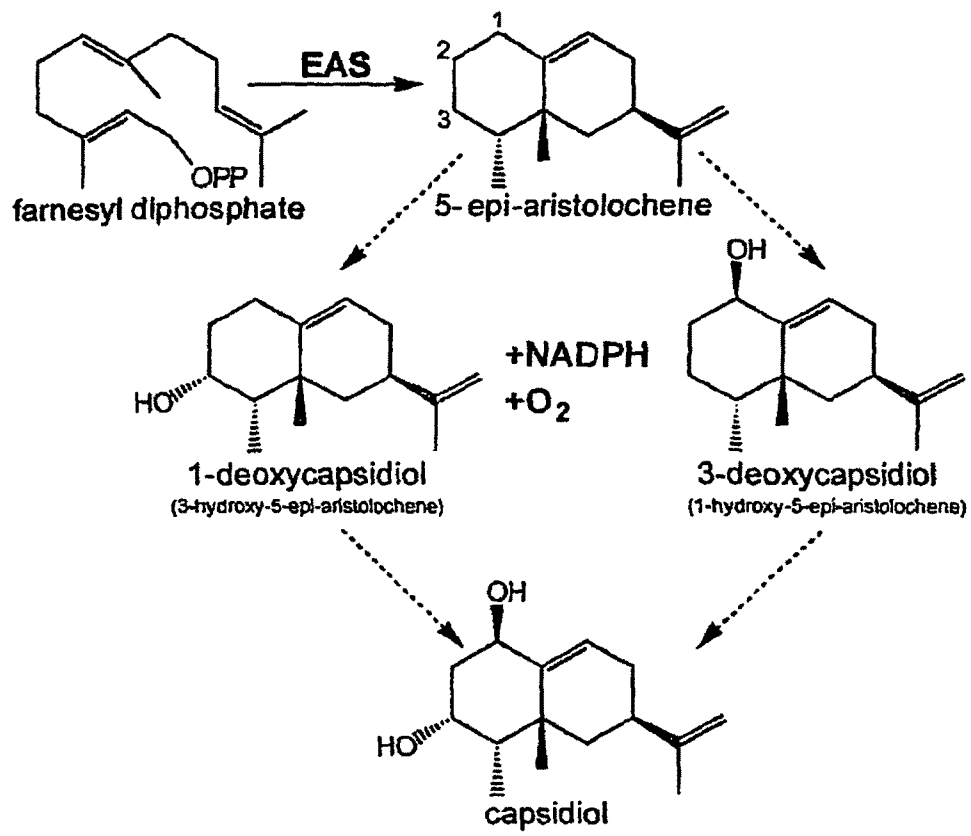


FIG. 2

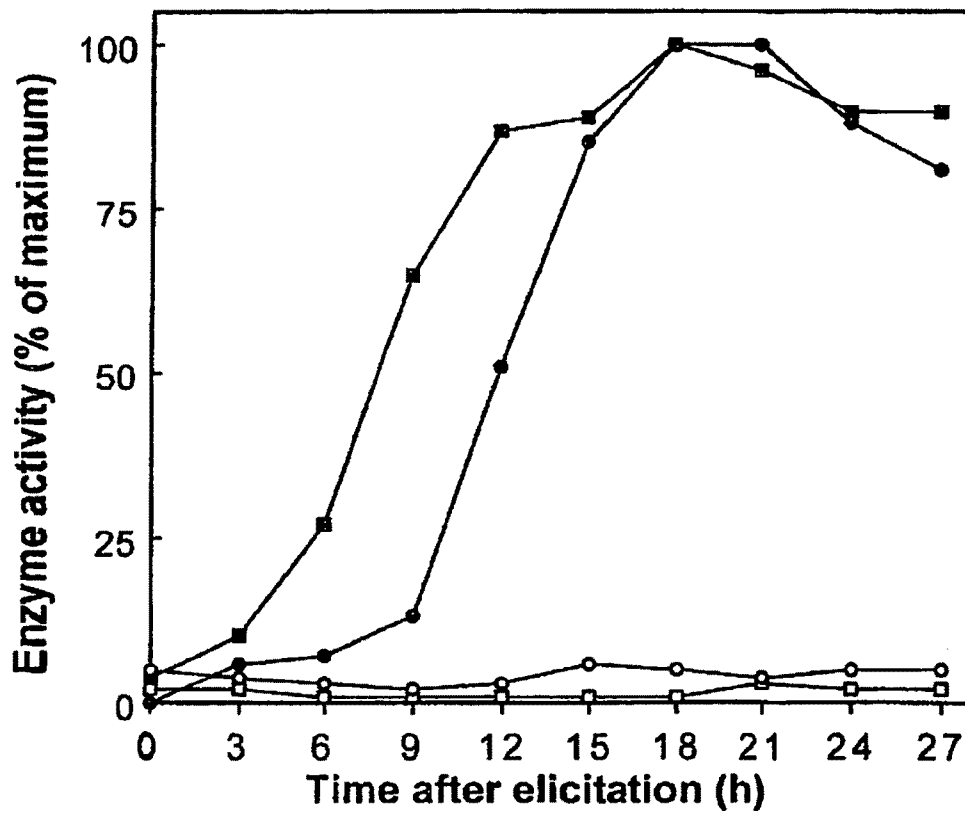


FIG. 3A

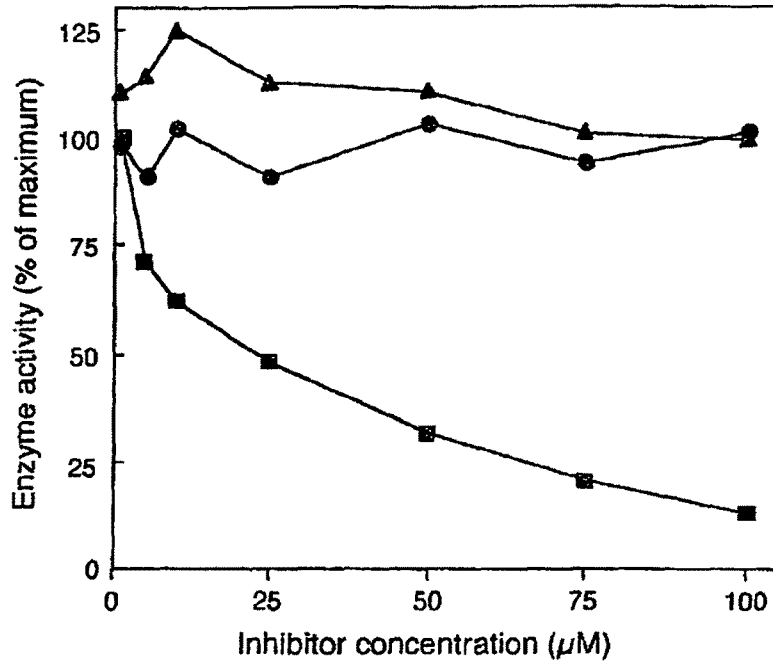


FIG. 3B

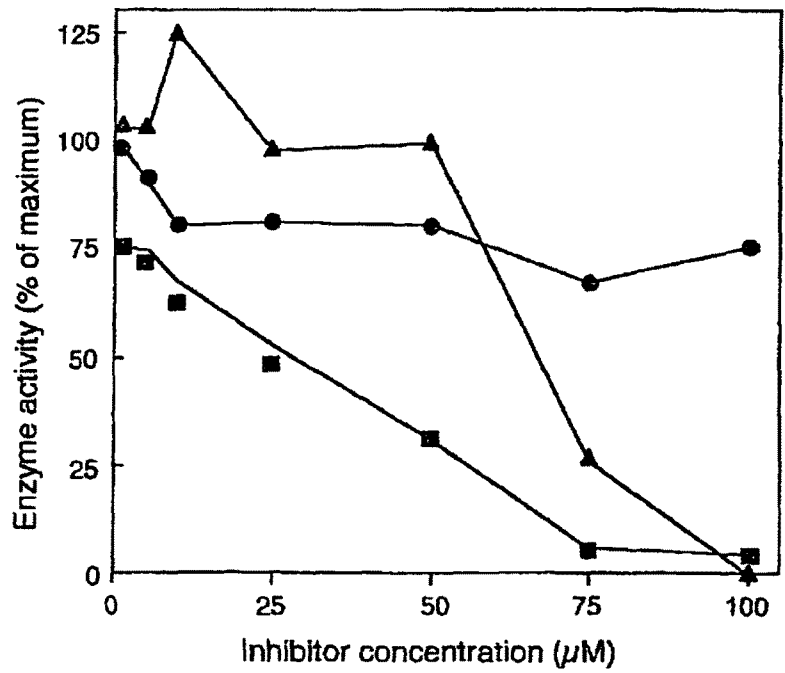


FIG. 4A

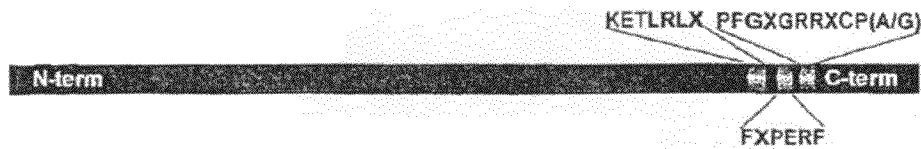


FIG. 4B

KETLRLH-for	5'-AARGARACIYTIMGIYTICA-3'
KETLRLY-for	5'-AARGARACIYTIMGIYTITA-3'
KETLRLR-for	5'-AARGARACIYTIMGIYTIMG-3'
FXPERF -for	5'-TTYIIICCI GARMGITY-3'
FXPERF-rev	5'-RAAICKYTCIGGIIIRAA-3'
GRRXCP(A/G)-for	5'-GGIMGIMGIIIIITGYCCIGS-3'
PFGXGRR-rev	5'-CKICKICCI III CCRAAIGG-3'
T7	5'-GTAATACGACTCACTATAGGG-3'
T3	5'-CAATTAACCCTCACTAAAGGG-3'

FIG. 4C

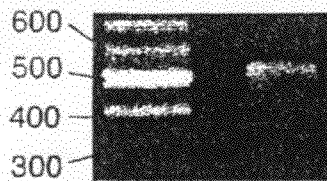


FIG. 6A

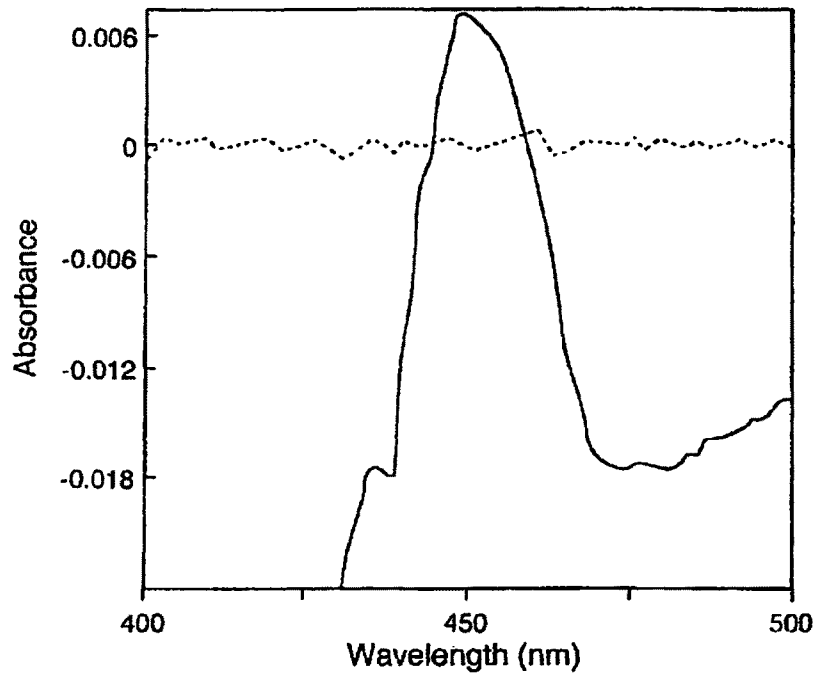
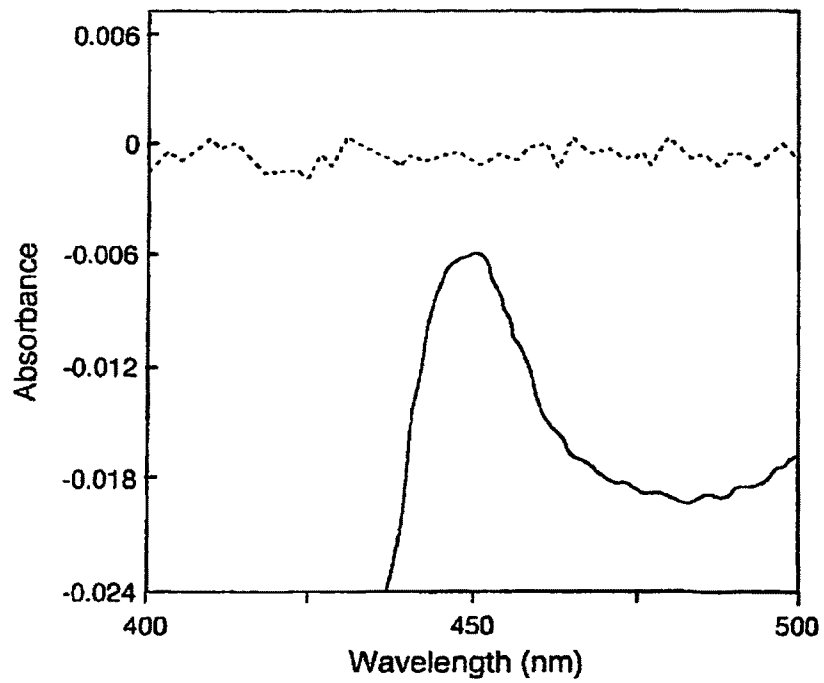
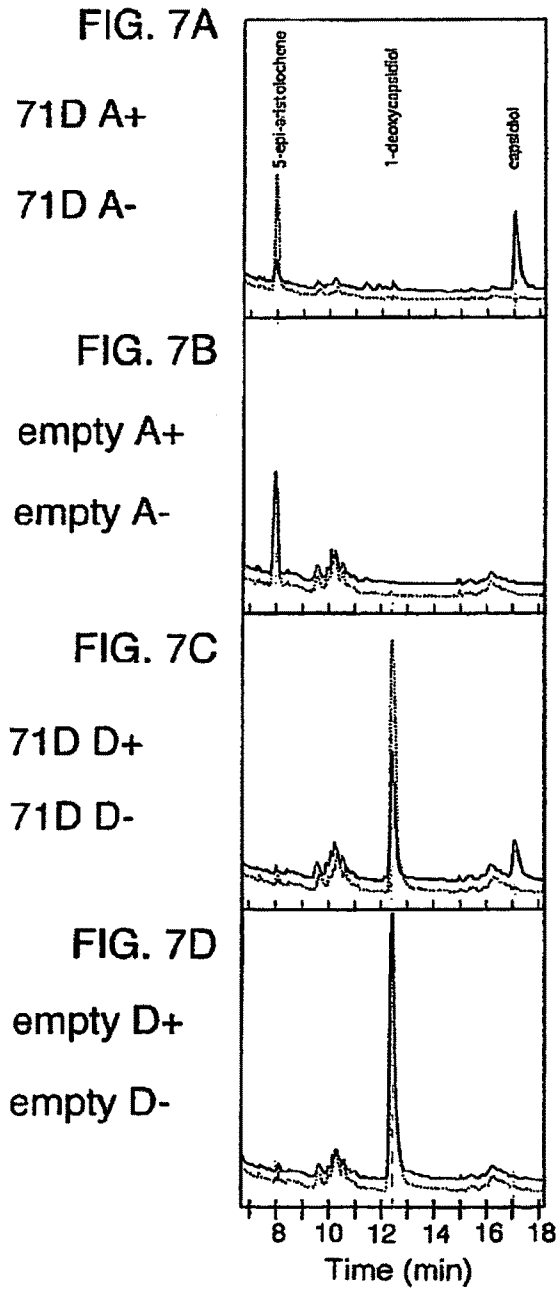


FIG. 6B





MpCYP71D13	M E L Q I S S A I I L V V T Y T I S L L I I K Q W R K P - K P Q - E	33
MsCYP71D18	M E L D L S A I I L V A T T Y I V S - L I L I M Q W R K S - K S Q - Q	32
NrCYP71A5v1	M A I M V S L S V F L I A L L C T T L P F L L F L N K K W R R S Y S G K T P P	33
PaCYP71A1	M A I L V S L L F L A I A L T F F L L L K N E K R E - - K K P P	29
NiCYP71D20	M Q F F S L V S I F F L F L S S F I F I F F F - L C R K W K N S - N S Q S K	30
AiCYP701A3	M A F F S M I S I L L G F V I L S L L N L S F S R K N M S E V S	39
MpCYP71D13	N L P P G P P - P K L P L G H L H L F L W G K L P Q H A L A S V A K Q Y G P V	70
MsCYP71D18	N L P P S P L - P K L P L G H L H L F L W G G L L P Q H V F R S I A Q K Y G P V	69
NrCYP71A5v1	- P P S P - P K L P L G H L H L F L W G L Y P Q H R Y L R Q S L S R R E Y G P L	67
PaCYP71A1	N L P P S P - P N L P L G H L H L F L W G - N L L P H R S L R S L A N E L G P L	65
NiCYP71D20	K L P P G P - W K L P L G H L H L F L W G M I G G E K K P H H V L R D L A K Y G P L	67
AiCYP701A3	T L P S V P V V R G F P V I G G N L L Q L H L L K E K P H H V L R D L A K Y G P I	78
MpCYP71D13	A H V Q L G E V F S V V L S S R I E A T K E A M K K L V D P A C A D R I F E S I G T	109
MsCYP71D18	A H V Q L G E V Y S V V L S S A E A A K Q A M K K V L D P N F A D R R F D G I G S	108
NrCYP71A5v1	M Q L H F G S V P T L V A S S P E A A R E I M K K N Q D I V F S N R P K M S I A	106
PaCYP71A1	I L L H L G H I P T L V V S T A E I A E E I L K T H D D I V F A S R P S T A A	104
NiCYP71D20	M H L Q L G E I S A V V T S R D M A K E V L K T T H D D V S S I S T R K I V A M	106
AiCYP701A3	Y S I K M G S S L I V L N S I E T G K E A M V T T R F S S I S T R K L S N A L	117
MpCYP71D13	K I M W Y D N D D I I F S P Y I S V H H W R Q M R K I C V S E L L S A R N V R S -	147
MsCYP71D18	R T M W Y D N D D I I F S P Y N D Y V H H W R Q M R I C V T E L L S P N V R S -	146
NrCYP71A5v1	N R L F F N R D V A F T Q Y G E Y W R Q I R S I C V L O L L S P N V R S -	144
PaCYP71A1	R R I F Y D C T D V A F S P Y G E Y W R Q I R K I C V L E L L S P N V R S -	142
NiCYP71D20	D I I C Y N Q S D I I A F S P Y G D D H W R Q M R K I C V M E L L S A R N V R S -	144
AiCYP701A3	T V L T C D K S M V A T S D Y D F H K L V R K I C V L L N G L L S A R N V R S -	155

Figure 8A

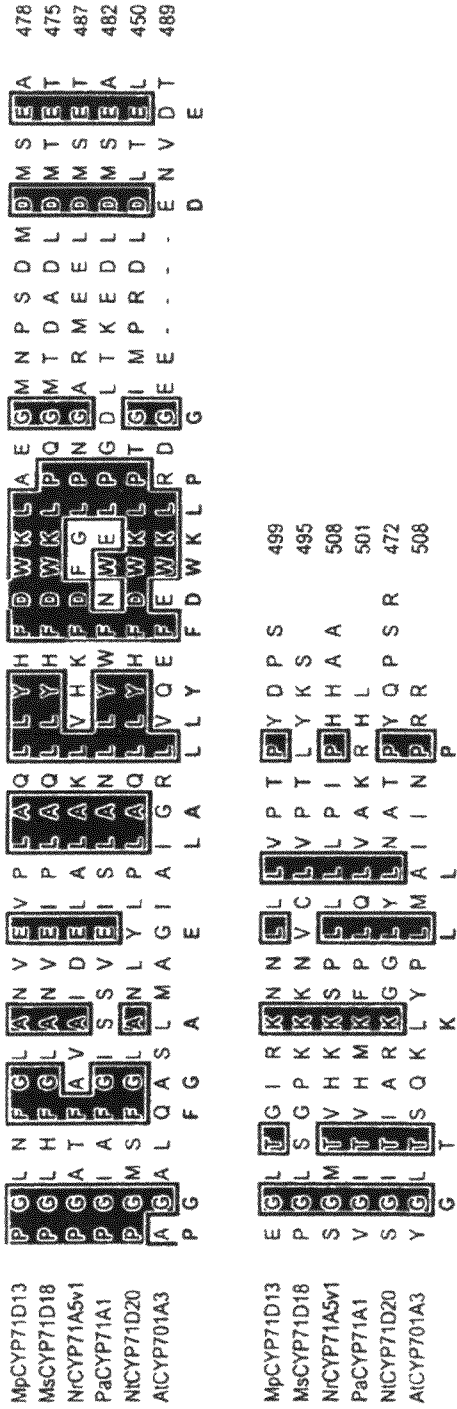


Figure 8D

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. 10/097,559, filed Mar. 8, 2002 (now issued U.S. Pat. No. 7,405,057), 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, in plants, cytochrome P450s play important roles in wound healing, pest resistance, signaling, and anti-microbial and anti-fungal activity.

Capsidiol is a bicyclic, dihydroxylated sesquiterpene produced by many Solanaceous species in response to a variety of environmental stimuli, including exposure to UV (Back 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 it 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; Back and Chappell, *Proc. Natl. Acad. Sci. U.S.A.* 93:6841-6845, 1996; Mathis et al., *Biochemistry* 36:8340-8348, 1997; Starks 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 capsidiol 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 capsidiol 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-deoxycapsidiol 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-deoxycapsidiol and reported a modest conversion of this compound to capsidiol 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 capsidiol when fed to elicitor-induced cell cultures but not control cultures. Whitehead et al. (*Phytochemistry* 28:775-779, 1989) there-

fore concluded that the 3-hydroxylase, responsible for hydroxylation of 5-epi-aristolochene at C3 to generate 1-deoxycapsidiol, was pathogen/elicitor inducible, while the 1-hydroxylase, responsible for hydroxylating 1-deoxycapsidiol at the C1 to generate capsidiol, 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 microsome preparations and subsequently determining the amount of 1-deoxycapsidiol 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-deoxycapsidiol was dependent on both

NADPH and O₂, and that 1-deoxycapsidiol accumulation in vitro was arrested by the P450 antagonists carbon monoxide (Omura and Sato, *J. Biol. Chem.* 239:2370-2378, 1964), ancyimidol (Coolbaugh et al., *Plant Physiol.* 62:571-576, 1978), and ketoconazole (Rademacher, *Arum. Rev. Plant Physiol. Plant Mol. Biol.* 51:501-531, 2000).

Recent results suggest that the hydroxylation of 5-epi-aristolochene is an important regulated step in capsidiol biosynthesis. In studies to evaluate the effectiveness of methyl jasmonate as an inducer of capsidiol 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 sesquiterpene cyclase reaction were limiting. Using an in vivo assay measuring the conversion rate of radiolabeled 5-epi-aristolochene to capsidiol, a very limited induction of the hydroxylase activity was observed in cells 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 capsidiol 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 (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 hydroxylating 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 isoprenoid 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 isoprenoid compound, the method including the steps of: (a) culturing a cell that expresses a recombinant isoprenoid synthase and a recombinant cytochrome P450 under conditions wherein the isoprenoid synthase and the cytochrome P450 are expressed and catalyze the formation of an isoprenoid compound not normally produced by the cell; and (b) recovering the isoprenoid 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 isoprenoid 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, hydroxylase activity, dual hydroxylase activity, demethylase 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 NOS:1, 3, 5, 7 and 11) or nucleic acid sequence (for example, the nucleic acid sequences shown in SEQ ID NOS:2, 4, 6, 8 and 12, respec-

tively). 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%, by weight, free from the proteins and naturally-occurring organic molecules with which it is naturally associated. Preferably, the preparation is at least 75%, more 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, 2×SSC, 10% Dextran sulfate, a first wash at approximately 65° C. in about 2×SSC, 1% SDS, followed by a second wash at approximately 65° C. in about 0.1×SSC. 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 transacetylase (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-dichloro isonicotinic 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 cell, as used herein includes, without limitation, algae, cyanobacteria, seeds, suspension cultures, embryos, meristematic 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 Agrios,

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

extracellular capsidiol, a dihydroxylated form of aristolochene (Mandujano-Chavez et al., Arch. Biochem. Biophys. 381 :285-294, 2000).

FIG. 3 is a series of graphs showing the dose dependent inhibition of 5-epi-aristolochene hydroxylase activity by ancyimidol and ketoconazole. Cell cultures were incubated in the presence of cellulase (0.5µg/ml) plus the indicated concentrations of ancyimidol (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 NOS:26-29).

FIG. 4B is a list of the degenerate P450-specific primers (SEQ ID NOS: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 T7 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 adsorption 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 WAT11 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 NtCYP71D20 (SEQ ID NO:1) with other plant terpene hydroxylases (SEQ ID NOS:39-43). NtCYP71A5v1 (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 MpCYP71D13 (AAD44151) catalyze the mono-hydroxylation at C6 and C3 of limonene, a cyclic monoterpene, respectively (Lupien et al., Arch. Biochem. Biophys. 368:181-192, 1999). AtCYP701A3 (AAC39505) encodes for kaurene oxidase, which catalyzes a 3-step reaction including a hydroxylation followed by oxidation of a diterpene (Helliwell et al., Plant Physiol. 119:507-510, 1999). Shown are sequences from *Mentha piperita* (MpCYP71D13; SEQ ID NO:39), *Mentha spicata* (MsCYP71D18; SEQ ID NO:40), *Nepeta racemosa* (NrCYP71A5v1; SEQ ID NO:41), *Nicotiana tabacum* (NtCYP71D20; SEQ ID NO:!), *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 Solanaceous 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, an in vivo assay for 5-epi-aristolochene hydroxylase-activity was developed and used to demonstrate a dose dependent inhibition of activity by ancyimidol 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 against 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 SEAH 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 SEAH, 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, SEAH 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 ancyimidol or ketoconazole were pre-incubated for 12 hours before measuring the cells' ability to convert exogenous supplied [³H] labeled 5-epi-aristolochene to radiolabeled capsidiol during a subsequent 3 hour incubation period (FIG. 3). Apparent activity of 5EAS was inhibited in a dose-dependent manner with approximately 50% inhibition by either 25μM ancyimidol or ketoconazole, and more than 80% by 75μM ancyimidol and 95% by 100 μM 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 ancyimidol 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 bp products were expected from reactions utilizing the primer prepared to the heme-binding domain (GRRXCP(A/G))(SEQ ID NOS:27 and 28) and the T7 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 DNAs contained signature sequences typical of P450 enzymes as revealed by BlastX database searches, and these corresponded to typical plant P450 family members of the CYP71, CYP73, 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 1 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

Full-length cDNAs cloned from an elicited cDNA library			
Cytochrome P450 cDNA clone	Nearest relative/ accession number	% Identity	% Similarity
CYP71D20	CYP71D7 (<i>S. chacoense</i>) Gen EMBL U48435	76.5	88.8
CYP71D21	CYP71D7 (<i>S. chacoense</i>) Gen EMBL U48435	76.3	88.8
CYP73A27	CYP73A15 (<i>P. vulgaris</i>) Gen EMBL Y09447	79.4	92.6

TABLE 1-continued

Full-length cDNAs cloned from an elicited cDNA library			
Cytochrome P450 cDNA clone	Nearest relative/ accession number	% Identity	% Similarity
CYP73A28	CYP73A15 (<i>P. vulgaris</i>) Gen EMBL Y09447	79.2	92.4
CYP82E1	CYP82E1 (<i>N. tabacum</i>) Gen EMBL AB015762	100.0	100.0
CYP92A5	CYP92A3 (<i>N. tabacum</i>) Gen EMBL X96784	95.5	98.6

The cloned fragments were used in a second step to isolate full-length clones 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, CYP82E1 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 EAS mRNA remained high throughout the duration of the experiment, the CYP73A27 mRNA was negligible in cells 24 hours after elicitor-treatment. In contrast, the CYP71D 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 ascribe functional identity to the various P450 cDNAs, full-length cDNAs for CYP71D20, CYP82E1 and CYP92A5 were inserted into the yeast expression vector pYedP60 (Urban et al., *Biochimie* 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 (Fahrendorf 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.*

U.S.A. 79:7410-7414, 1982), and expression was compared to yeast transformed with the parent pYeDP60 vector (control) alone.

After induction with galactose for approximately 16 hours, control cells and cells containing the various P450 constructs were collected, and microsomes prepared from each were analyzed for general P450 expression by CO-difference spectroscopy (Omura and Sato, J. Biol. Chem. 239:2370-2378, 1964). Microsomes prepared from cells containing the CYP71D20 (FIG. 6A) and CYP92A5 (FIG. 6B) constructs both showed characteristic CO difference spectra with peaks at 450 nm, indicating that the encoded proteins were assembling properly with their heme cofactor. Using the extinction coefficient of $91 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ for heme binding proteins (Omura and Sato, J. Biol. Chem. 239:2370-2378, 1964), it was determined that approximately 107 pmol of CYP71D20 and 268 pmol of CYP92A5 were expressed in the yeast cells per milligram of total yeast protein.

Both 5-epi-aristolochene and 1-deoxycapsidiol were metabolized to only one product with the same retention time as capsidiol. Obvious by its absence, no reaction product having a retention time similar to deoxycapsidiol was detectable in the 5-epi-aristolochene incubations (FIG. 7). Co-injection of authentic capsidiol with the respective reaction products resulted in a single GC peak having a 16.2 minute retention time, identical to capsidiol. Mass spectra patterns for the separate reaction products were identical to that for the capsidiol standard (EIMS m/z 236, 221, 203, 185, 175, 163, 157, 133, 121, 107, 93, 79, 67, 55, 43, 41).

The in vivo assay data presented in FIGS. 2 and 3 of the current work indicate that the conversion of 5-epi-aristolochene is catalyzed by at least one inducible cytochrome P450 mediated reaction.

Furthermore, any of the cytochrome p450 polypeptides described herein may include one or more hydroxylase activities which can incorporate hydroxyl groups into at least two distant sites on an isoprenoid compound. The addition of these hydroxyl groups may occur, for example, sequentially, by adding a hydroxyl group first to one site and then the other, in either order. Moreover, such hydroxylases may be mutated to limit their ability to hydroxylate a substrate at only one site, or, alternatively, to provide stereochemical specificity to their hydroxylating activity.

The above-described experiments were performed using the following materials and methods.

Chemicals

Standard laboratory reagents were purchased from Becton Dickinson Microbiology Systems (Sparks, Md.), FisherBiotech (Fair Lawn, N.J.) and Sigma Chemical Company (St. Louis, Mo.).

Biological Materials and Induction Treatments

Nicotiana tabacum cv. KY14 plants and cell suspension cultures were used. Cell suspension cultures were maintained in modified Murashige-Skoog (Vogeli and Chappell, Plant Physiol. 88:1291-1296, 1988). Cultures in their rapid phase of growth (3 days old) were used for all experiments. At the indicated times, cells were collected and separated from media by vacuum filtration and stored at -80°C .

Induction treatments were performed by the addition of the fungal elicitors, cellulase (*Trichoderma viride*, Type RS, Onozuka) or paraciticein (O'Donohue et al., Plant Mol. Biol. 27:577-586, 1995) at the indicated concentrations. Paraciticein was purified from *E. coli* cells overexpressing a recombinant paraciticein protein containing a carboxy-terminal histidine purification tag.

In vivo 5-Epi-aristolochene Hydroxylase Assay and Inhibition Studies

5-epi-aristolochene hydroxylase-activity was measured as the incorporation of [^3H]-5-epi-aristolochene into extracellular capsidiol by intact cells. [^3H]-5-epi-aristolochene was produced by incubating an excess of [^3H] farnesyl diphosphate ($1 \mu\text{M}$, 20.5 Ci/mmol) with recombinant 5-epi-aristolochene synthase (Back 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 FPP before quantifying the yield of radioactive 5-epi-aristolochene by liquid scintillation counting. The hexane solvent was removed under a gentle stream of N_2 gas, and the dried residue was re-dissolved in acetone. Control and elicitor-treated cells were then incubated with [^3H]-5-epi-aristolochene (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 ancymidol (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 cellulase ($0.5 \mu\text{g}/\text{ml}$) and indicated concentrations of ancymidol or ketoconazole for 12 hours prior to the addition of [^3H]-5-epi-aristolochene. 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 Vogeli 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 ancymidol 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 ($0.5 \mu\text{g}$ 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 μ l aliquot of the elicitor-induced cDNA library (3×10^6 pfu/ μ l) by heat denaturation at 70° C. for 10 minutes, followed by phenol/chloroform extraction, ethanol precipitation and re-suspension in 500 μ l of sterile, deionized water. Amplification reactions were performed in 50 μ l volumes containing 50 mM KCl; 10 mM Tris-HCl, pH 8.8; 1.5 mM MgCl₂; 200 μ M of each dNTP; 2 μ l 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, WI). Resulting recombinant plasmids containing insert DNAs within the expected size range were sequenced using T7 and Sp6 primers.

DNA Sequencing

All the DNA sequencing reactions were performed using the BIGDYE™ 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 by . DNA fragment amplified from the pGEM-deg6.4 clone using gene-specific forward (5'-GGCGGAGAATTGTCCTGGAATGTCATTTGGTTTAG-3' (SEQ ID NO:13)) and reverse (5'-GTACAATAGTGAGGTTGACAATG-3' (SEQ ID NO:14)) primers; and a 374 by DNA fragment amplified from the pBKS-CYPB3.843 clone with specific forward (5'-GGTG-GTTGTGAATGCATG-3' (SEQ ID NO:15)) and reverse (5'-TTATGCAGCAATAGGCTTGAAGACA-3' (SEQ ID NO:16)) primers, were used to screen for CYP71Ds. The probes were labeled with digoxigenin-11-dUTP using the PCR DIG Labeling Mix (Roche Molecular Biochemicals, Indianapolis, Ind.), hybridized to plaque lifts 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.). Plaques exhibiting 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 μ g 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 rRNA 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 2xSSC/0.1% SDS once at room temperature followed by sequential washes in 0.2xSSC/0.1% SDS at 42° C.

and 65° C. Hybridization was detected with a Phosphorimager (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. 272:19176-19186, 1990; Pompon et al., Methods Enzymol. 272:51-64, 1996). Appropriate BamHI, EcoRI, and SstI 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'-GGGGGATCC ATGCAATTCTTCAGCTTG-GTITCC-3' (SEQ ID NO:17) and 5'-GGGGAATC TTACTCTCGAGAAGGTTGATAAGG-3' (SEQ ID NO:18); for the CYP82E1 cDNA 5'-CCCGGATCC ATGATATCATCT-TCTTTCCTCCC-3' (SEQ ID NO:19) and 5'-GGGGAATC TCAATATTGATAAAGCGTAGGAGG-3' (SEQ ID NO:20); and for the CYP92A3 cDNA 5'-CCCGGATCC ATGCAATC-CTTCAGCTTGGTTTCC-3' (SEQ ID NO:21) and 5'-GGG GAGCTC TCACTCGCAAGAAGATTGATAAGG-3' (SEQ ID NO: 22). Two long, overlapping (italicized) primers 5'-GCCATATCGGCGCAATACTAATCTC-CAAACTCCGCGGT AAAAAATTCAAGC TCCCAC-CTGGTCCAACAGCAGTC-3' (SEQ ID NO:23) and 5'-GGGGGATCC ATGGACCTCCTCCTCATA-GAAAAAACCCTCGTCGCCTTATTC GCCGCCAT-TATCGGCGCAATACTA-3' (SEQ ID NO:24) coding for the N-terminal sequence 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'-GGGGAGCTC TTATGCAG-CAATAGGCTTGAAGAC-3' (SEQ ID NO:25). CYP71D20 and CYP73A27 were amplified using full-length cDNA templates, whereas CYP82E1 and CYP92A5 were amplified directly from the cDNA library template. Amplifications were performed in 50 μ l reactions containing 1xPfx amplification buffer; 1 mM MgSO₄; 300 μ M 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

Verified pYeDP60-P450 cDNA constructs were introduced into the yeast WAT11 line, a derivative of the W303-1B strain (MAT a; ade 2-1; his 3-11; leu 2-3,-112; ura 3-1; can^R; cyr⁺), provided by Dr. P. Urban (Centre de Génétique Moléculaire, CNRS, Gif-sur-Yvette, France). The endogenous NADPH-cytochrome P450 reductase (CPR1) locus has been replaced with ATR1, a NADPH-cytochrome P450 reductase from *Arabidopsis thaliana* (Pompon et al., Methods Enzymol. 272:51-64, 1996; Urban et al., J. Biol. Chem. 272: 19176-19186, 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 μ l of yeast suspension was mixed with 0.5 to 1

μ g plasmid DNA (in $<5 \mu$ l dH_2O) in a pre-chilled 0.5 ml tube, and transferred to a chilled cuvette with a 0.2 cm electrode gap. One pulse at 1.5 kV, 25 μ F, and 200 Ohms was applied by an Eppendorf Electroporator (model 2510). A mixture of 500 μ l of YPAD/1M sorbitol was immediately added to the electroporated cells. Cells were allowed to recover at 30° C. for 1 hour, then spread onto SGI plates (1 g/l bactocasamino 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 bactocasamino 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 bacto-peptone; 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 μ l 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 \times g for 10 minutes. The pelleted cells were washed with 100 ml of TES buffer (50 mM Tris-HCl, 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 \times g for 10 minutes, and the pellet was resuspended in 2.5 ml extraction buffer (1% bovine serum albumin, fraction V; 2 mM 2-mercaptoethanol; 1 mM phenylmethylsulfonyl fluoride, all dissolved in TES). Glass beads (0.5 mm in diameter, Biospec Products, Inc., Bartlesville, Okla.) were added until skimming the surface of the cell suspension. Cell walls were disrupted manually by hand shaking in a cold room for 10 min at 30 second intervals separated by 30 second intervals on ice. 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 extracts. 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 \times g for 70 minutes at 4° C., and microsomal pellets were resuspended in 1.5 ml TEG-M buffer (50 mM Tris-HCl, 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

$\text{Fe}^{2+}\cdot\text{CO}$ vs. Fe^{2+} difference spectroscopy (Omura 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-HCl, 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 $\text{mM}^{-1}\cdot\text{cm}^{-1}$.

5-Epi-aristolochene-1,3-hydroxylase Assays

5-epi-aristolochene-1,3-hydroxylase assays were performed in 0.5 ml polyethylene tubes in 100 μ l volumes. 5-epi-

aristolochene or 1-deoxycapsidiol 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-deoxycapsidiol were resuspended in 2 μ l dimethyl sulfoxide before adding the reaction mixture. Reactions were carried out in 100 mM Tris-HCl, 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-deoxycapsidiol in these assays varied from 20 to 50 μ M. 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-deoxycapsidiol (Whitehead et al., *Phytochemistry* 29:479-182, 1990), and capsidiol (Whitehead et al., *Phytochemistry* 26:1367-1369, 1987; Milat 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 $\text{m}\times$ 0.25 mm, 0.25 μ m 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 $\text{m}\times$ 0.25 mm, 0.25 μ m phase thickness) and run with He 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 based 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 hybridizing 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 Rapid 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, Grape, Arabidopsis, Lettuce, Sunflower, Oilseed rape, Flax, Cotton, Sugarbeet, Celery, Soybean, Alfalfa, Medicago, Lotus, Vigna, Cucumber, Carrot, Eggplant, Cauliflower, Horseradish, Morning Glory, Poplar, Walnut, Apple, Asparagus, Grape, Rice, Maize, Millet, 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 I, 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 most 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*) (Goedel et al., Nucl. Acids Res. 8:4057, 1980), and the *tac* promoter systems, as well as the lambda-derived P.sub.L 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* pET 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 reductase are described in Porter et al. (Drug Metab. Rev. 31:159-174, 1999).

For eukaryotic expression, the method of transformation or transfection and the choice of vehicle for expression of the P450 will depend on the host system selected. Transformation and transfection methods of numerous organisms, for example, the baker's yeast *Saccharomyces cerevisiae*, are described, e.g., in Ausubel et al. (supra); Weissbach and Weissbach, Methods for Plant Molecular Biology, Academic Press, 1989; Gelvin et al., Plant Molecular Biology Manual, Kluwer Academic Publishers, 1990; Kindle, K., Proc. Natl. Acad. Sci. U.S.A. 87:1228 (1990); Potrykus, I., Arum. Rev. Plant Physiol. Plant Mol. Biology 42:205 (1991); and BioRad (Hercules, Calif.) Technical Bulletin #1687 (Biolistic Particle Delivery Systems). Expression vehicles may be chosen from those provided, e.g., in Cloning Vectors: A Laboratory Manual (P. H. Pouwels et al., 1985, Supp. 1987); Gasser and Fraley (supra); Clontech Molecular Biology Catalog (Catalog 1992/93 Tools for the Molecular Biologist, Palo Alto, Calif.); and the references cited above.

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 a 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 vehicle include COS cells and CHO cells (ATCC Accession Nos. CRL 1650 and CCL 61, respectively).

Alternatively, if desired, a 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 Pouwels 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 0.01-300 μ M 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 pCVSEII-DHrF and pAdD26SV(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 (SO-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 Pouwels 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 caulimovirus 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 35S and 19S promoters. In most tissues of transgenic plants, the CaMV 35S 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., Dekeyser et al., *Plant Cell* 2:591, 1990; Terada 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 35S 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 Kuhlemeier 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 *Em* gene of wheat described by Marcotte et al. (*Plant Cell* 1:969, 1989); the ABA-inducible *HVA1* and *HVA22*, and the *rd29A* 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 *wund* 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 Roshal et al. (*EMBO J.* 6: 1155, 1987); the 23-kDa zein gene from maize described by Scherthauer et al. (*EMBO J.* 7:1249, 1988); or the French bean beta-phaseolin gene described by Bustos 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' terminator region may be included in the expression vector to increase stability of the mRNA. One such terminator region may be derived from the PI-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 those cells that have become transformed. Useful selectable genes for plant systems include genes encoding antibiotic resistance genes, for example, those encoding resistance to hygromycin, kanamycin, bleomycin, G418, streptomycin, or spectinomycin. Genes required for photosynthesis may also be used as selectable markers in photosynthetic-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, especially 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

Upon 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*; Dekeyser et al., *supra*; Fromm et al., *Nature* 319:791, 1986; Sheen,

Plant Cell 2:1027, 1990; or Jang 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 Agrobacterium 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 Agrobacterium and a high copy number origin of replication functional in *E. coli*. This permits facile production and testing of transgenes in *E. coli* prior to transfer to Agrobacterium for subsequent introduction into plants. Resistance genes can be carried 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 endonuclease sites for the addition of one or more transgenes and directional T-DNA border sequences which, when recognized 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 Biolistic 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 DNA has been precipitated is placed on the front of the plastic macroprojectile. 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 agricultural 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 techniques. 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. Transformation 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., 100 µg/ml). Kanamycin-resistant shoots are then placed on plant tissue culture media without hormones for root initiation. Kanamycin-resistant plants are then selected for green house growth. If desired, seeds from self-fertilized transgenic plants can then be sowed 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 accomplished 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. Consequently, 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 transgene 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 hybridization and immunocytochemistry according to standard protocols 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 or 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 fractionation 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, Laboratory Techniques in Biochemistry and Molecular Biology, eds., Work and Burdon, Elsevier, 1980).

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-pathogenic 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 monoterpenes, 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 methylase transferase 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.

SEQUENCE LISTING

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<210> SEQ ID NO 4

<211> LENGTH: 1614

<212> TYPE: DNA

<213> ORGANISM: Nicotiana tabacum

<400> SEQUENCE: 4

ggatggtcta ataatcctcc atttatctcc caaaatggaa ttcttcagct tggtttccat

60

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attcctatc ctatctttcc tctttttggt aaggaaatgt aagaactcca atagcctaac 120
caacaattg cctccaggtc catggaaaat accaatacta ggaagtatgc ttcatatgct 180
tggaggagaa ccacaccata tccttaggga tttagccaaa aaatatggac caattatgca 240
ccttcagttt ggtgaaattt ctgcagttgt ggttacttct agggagatgg caaagaagt 300
gctaaaaact catgacgtag tttttgcatc taggcctaaa attgtggcca tggacattat 360
ctgttataac cagtctgata tcgcttttag cccttatggc gatcactgga gacaaatgag 420
taaaatttgt gtcacggaac ttctaatgc aaagaatgtt cggcttttca gctcagatcag 480
acgtgatgaa gtcgttcgtc tcattgactc tattcgatca gattcttctt ctggtgagct 540
agttaatttt acgcaaggga tcatttggtt cgcgagctcc atgacgtgta gatcagcatt 600
tgggcaagta cttagggggc aagacgtatt tgccaaaaag attagagaag taatagggtt 660
agcagaaggc tttgatgtgg ccgatatctt cccttcatac aagtttcttc atgttctcag 720
tggaatgaag cgtaaacctc tgaatgccc ccttaaggta gatgccattg ttgaggatgt 780
catcaacgag cacaagaaaa atcttgcaac tgggaaaact aatggagcat taggagacat 840
gtttgctgcc ggaacagaaa ctctcatcaac aacaactgta tgggctatgg ctgaaatgat 900
gaagaatcca aatgtattca acaaagctca ggcagaatg agagaaacct ttaaagacaa 960
agtaacattt gatgaaattg atgcagagga gctggaatac ttaaagttag ttattaaaga 1020
aactttgaga ctctaccac cgtctccact tttggtccca agagaatgta ggaagatac 1080
agatattaac ggctacacta ttcctgcgaa gaccaaagtt atggttaatg tttgggcatt 1140
gggaagagat ccaaaatatt gggatgacgc agaaagcttt aagccagaga gatttgagca 1200
atgctctgtg gatttttttg gtaataattt tgagtttctt ccctttggcg gtggacggag 1260
aatatgtcct ggtatgtcat ttggtttagc taatctttac ttgccattgg ctcaattgct 1320
atatcacttt gattggaaac tcccagcgg aatgatgccc ggagacttgg acttgactga 1380
attagctgga ataacaattg ctagaagggt tgacctttac ttaatggcta ctcttatca 1440
accttctcgc gaataattta atggcatcag gttttttaat tccattgtca acctcactat 1500
tgtacaagct ttctgatgtt tcaggttttg ccgatttgta ataatgtag tttttataat 1560
atgtatcata cccatgtaga agagggacga ttaattagtt gtaaaaaaaaa aaaa 1614

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<210> SEQ ID NO 5

<211> LENGTH: 537

<212> TYPE: PR

<213> ORGANISM: Nicotiana tabacum

<400> SEQUENCE: 5

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Met Lys Asn Met Ala Lys Leu Leu Asn Lys Thr Ile Phe Cys Ile Leu
1           5           10           15
Phe Thr Ile Ala Phe Leu Ser Phe Ala Lys Leu Leu Ser Ser Tyr Leu
20           25           30
Ser Met Pro Phe Pro Leu Lys Tyr Met Ser Leu Ile Val Pro Leu Leu
35           40           45
Pro Leu Ile Ile Asn Phe Leu Tyr Val Lys Pro Gln Asn Asn Leu Pro
50           55           60
Pro Gly Pro Thr Ala Val Pro Ile Phe Gly Asn Trp Leu Gln Val Gly
65           70           75           80
Asn Asp Leu Asn His Gln Leu Leu Ala Thr Met Ser Gln Thr Tyr Gly
85           90           95
Pro Ile Phe Leu Leu Lys Leu Gly Ser Lys Asn Leu Ala Val Val Ser

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100					105					110					
Asn	Pro	Glu	Leu	Ala	Asp	Gln	Val	Leu	His	Thr	Gln	Gly	Val	Glu	Phe
		115					120					125			
Gly	Ser	Arg	Pro	Arg	Asn	Val	Val	Phe	Asp	Ile	Phe	Thr	Gly	Asn	Gly
		130					135					140			
Gln	Asp	Met	Val	Phe	Thr	Ile	Tyr	Gly	Asp	His	Trp	Arg	Lys	Met	Arg
		145					150					155			
Arg	Ile	Met	Thr	Leu	Pro	Phe	Phe	Thr	Asn	Lys	Val	Val	His	Gln	Tyr
				165					170					175	
Ser	Asp	Met	Trp	Glu	Asn	Glu	Met	Asp	Leu	Val	Val	Asn	Asp	Leu	Lys
				180					185					190	
Lys	Asn	Glu	Lys	Val	Lys	Tyr	Glu	Gly	Ile	Val	Ile	Arg	Lys	Arg	Leu
		195					200					205			
Gln	Leu	Met	Leu	Tyr	Asn	Ile	Met	Tyr	Arg	Met	Met	Phe	Asp	Ala	Lys
		210					215					220			
Phe	Glu	Ser	Gln	Asn	Asp	Pro	Leu	Phe	Ile	Glu	Ala	Thr	Lys	Phe	Asn
		225					230					235			
Ser	Glu	Arg	Ser	Arg	Leu	Ala	Gln	Ser	Phe	Asp	Tyr	Asn	Tyr	Gly	Asp
				245					250					255	
Phe	Ile	Pro	Leu	Leu	Arg	Pro	Phe	Leu	Arg	Gly	Tyr	Leu	Asn	Lys	Cys
				260					265					270	
Lys	Asp	Leu	Gln	Thr	Arg	Arg	Leu	Ala	Phe	Phe	Asn	Asn	Tyr	Phe	Val
		275					280						285		
Glu	Lys	Arg	Arg	Lys	Ile	Met	Asp	Glu	Asn	Gly	Glu	Lys	His	Lys	Ile
		290					295					300			
Ser	Cys	Ala	Ile	Asp	His	Ile	Ile	Asp	Ala	Glu	Met	Lys	Gly	Glu	Ile
		305					310					315			
Asn	Glu	Gln	Asn	Val	Leu	Tyr	Ile	Val	Glu	Asn	Ile	Asn	Val	Ala	Ala
				325					330					335	
Ile	Glu	Thr	Thr	Leu	Trp	Ser	Met	Glu	Trp	Ala	Ile	Ala	Glu	Leu	Val
				340					345					350	
Asn	His	Pro	Ile	Val	Gln	Gln	Lys	Ile	Arg	Asp	Glu	Ile	Ser	Thr	Val
		355					360					365			
Leu	Lys	Gly	Arg	Ser	Val	Thr	Glu	Ser	Asn	Leu	His	Glu	Leu	Pro	Tyr
		370					375					380			
Leu	Leu	Ala	Thr	Val	Asn	Glu	Thr	Leu	Arg	Leu	His	Thr	Pro	Ile	Pro
		385					390					395			
Leu	Leu	Val	Pro	His	Met	Asn	Leu	Glu	Glu	Ala	Lys	Leu	Gly	Gly	Tyr
				405					410					415	
Thr	Ile	Pro	Lys	Glu	Thr	Lys	Val	Val	Val	Asn	Ala	Trp	Trp	Leu	Ala
				420					425					430	
Asn	Asn	Pro	Ala	Trp	Trp	Lys	Asn	Pro	Asn	Glu	Phe	Arg	Pro	Glu	Arg
		435					440					445			
Phe	Leu	Glu	Glu	Asp	Ser	Ser	Thr	Glu	Ala	Ala	Val	Ala	Gly	Gly	Lys
		450					455					460			
Val	Asp	Phe	Arg	Tyr	Leu	Pro	Phe	Gly	Met	Gly	Arg	Arg	Ser	Cys	Pro
		465					470					475			
Gly	Ile	Ile	Leu	Ala	Leu	Pro	Ile	Leu	Gly	Leu	Val	Ile	Ala	Lys	Leu
				485					490					495	
Val	Ser	Asn	Phe	Glu	Met	Gln	Gly	Pro	Pro	Gly	Val	Glu	Lys	Val	Asp
				500				505						510	
Thr	Ser	Glu	Arg	Gly	Gly	Gln	Phe	Ser	Leu	His	Ile	Ala	Lys	His	Ser
				515			520							525	

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Thr Val Val Phe Lys Pro Ile Ala Ala
530 535

<210> SEQ ID NO 6
<211> LENGTH: 1745
<212> TYPE: DNA
<213> ORGANISM: *Nicotiana tabacum*

<400> SEQUENCE: 6

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cctctagcta atgaaaaaca tggccaaact tctcaacaag accatctttt gcattctctt    60
tacaattgca tttctttcat ttgccaaagt actgtcctcc tacctatcta tgcctttccc    120
tcttaagtac atgtcactta ttgtcccttt acttcccctt ataatcaact tcctctatgt    180
taagccccaa aacaacctcc cacctgggtcc aacagcagtc ccaatatttg gtaattgggt    240
tcaagttggc aatgacttga accatcaact ccttgccacc atgtcacaaa cctacgggtcc    300
tatattttta ctcaactctg gttcaaaaaa cctagctgtg gtatcaaac cagagctagc    360
tgaccaagtt ctacacacac aaggggtcga gtttgggtcc cgtccacgta acgttgtctt    420
cgacatattt actggtaatg gacaagacat ggtgttcacc atttatggtg accattggcg    480
aaaaatgagg cgtattatga cgcttcocatt tttcactaac aaagtgggtc accaatatag    540
tgatatgtgg gagaatgaga tggacttagt tgtaaatgac ttgagaaga atgaaaaagt    600
gaaatgatgag ggaattgtga ttaggaaacg attgcagctg atgctgtata acatcatgta    660
tcgaatgatg tttgatgcca aatttgagtc ccaaatgat cctttgttca ttgaggcaac    720
aaagttaat tcagagagaa gcagattagc tcagagcttt gactacaatt atggtgattt    780
tatcccttta cttagaccat tcttgagagg gtaccttaac aagtgtaaag acttacaac    840
aaggagactt gcattcttca acaattattt tgtagagaaa agaaggaaaa taatggatga    900
aaatggagaa aagcataaga taagctgtgc tattgatcac attatagatg ccgaaatgaa    960
aggagaataa aatgacaaa atgtactcta tatttgaggag aatatcaatg ttgcagcaat   1020
tgaacaact ctatggtcca tggaaatggc catagctgaa cttgtaaatc atcccattgt   1080
tcaacagaag attagggatg aaatctcaac agtcctcaa ggagatcag tcacagaatc   1140
aaacctccat gagctgcctt acttgctagc aacagtaaat gaaacattaa gactccacac   1200
accaatacct ttactgtac cccatagaa ccttgaagaa gcaaagttag gtggttacac   1260
tattcctaaa gaaactaagg tgggtgtgaa tgcgtgggtg ctggctaaca acctgcttg   1320
gtggaaaaac ccgaatgaat tccgcccga gaggtttctt gaggaggata gtagcacaga   1380
ggcagctggt gctggtggca aggtagattt caggtactta ccattcggta tggggaggcg   1440
gagctgcccc ggaatcatcc ttgcactgcc aattctgggg cttgtcatag ccaaactggt   1500
gtcaaatttt gaaatgcagg gtccaccagg tgtggaaaag gttgatacaa gtgaaagagg   1560
agggcagttt agcttgcaaa ttgcaaaaac ttccacggtt gtcttcaagc ctattgctgc   1620
ataataatat gcttaagcta tccttgtttt aattatattt gtcttaccag aaagcaaac   1680
tactaagtta ctcgataaag atttcaatga atattacagt ttttgttaaa aaaaaaaaaa   1740
aaaaa                                             1745
```

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

<400> SEQUENCE: 7

Met Ala Lys Leu Leu Asn Asn Thr Ile Phe Cys Ile Leu Phe Ser Ile

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1	5	10	15
Val Phe Leu Ser Phe Ala Lys Leu Leu Ser Ser Tyr Leu Ser Ile Pro	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 Gln Asn Asn Leu Pro Pro Gly Pro	50	55	60
Thr Ala Val Pro Ile Phe Gly Asn Trp Leu Gln Val Gly Asn Asp Leu	65	70	75
Asn His Gln Leu Leu Ala Thr Met Ser Gln 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 Gln Val Leu His Thr Gln Gly Val Glu Phe Gly Ser Arg	115	120	125
Pro Arg Asn Val Val Phe Asp Ile Phe Thr Gly Asn Gly Gln Asp Met	130	135	140
Val Phe Thr Ile Tyr Gly Asp His Trp Arg Lys Met Arg Arg Ile Met	145	150	155
Thr Leu Pro Phe Phe Thr Asn Lys Val Val His Gln 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 Gln 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	225	230	235
Ser Arg Leu Ala Gln Ser Phe Asp Tyr Asn Tyr Gly Asp Phe Ile Pro	245	250	255
Leu Leu Arg Pro Phe Leu Lys Gly Tyr Leu Asn Lys Cys Lys Asp Leu	260	265	270
Gln Thr Arg Arg Leu Ala Phe Phe Asn 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	290	295	300
Ile Asp His Ile Ile Asp Ala Glu Met Lys Gly Glu Ile Ser Glu Gln	305	310	315
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 Gln Gln Lys Ile Arg Asp Glu Ile Ser Thr Val Leu Lys Gly	355	360	365
Lys Ser Val Lys Glu Ser Asn Leu His Glu Leu Pro Tyr Leu Leu Ala	370	375	380
Thr Val Asn Glu Thr Leu Arg Leu His Thr Pro Ile Pro Leu Leu Val	385	390	395
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 Val Asn Ala Trp Trp Leu Ala Asn Asn Pro	420	425	430

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Ala Trp Trp Lys Asn Gln Asn Glu Phe Arg Pro Glu Arg Phe Leu Glu
 435 440 445

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 Asn
 485 490 495

Phe Glu Met Gln Ala Pro Pro Gly Val Gly Lys Val Asp Thr Ser Glu
 500 505 510

Lys Gly Gly Gln Phe Ser Leu His Ile Ala Lys His Ser Thr Val Val
 515 520 525

Phe Lys Pro Ile Ala Ala
 530

<210> SEQ ID NO 8
 <211> LENGTH: 1693
 <212> TYPE: DNA
 <213> ORGANISM: Nicotiana tabacum

<400> SEQUENCE: 8

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 ttcaattgta tttctttcat ttgccaaatt actatcctcc tacctctcta tacctttccc 120
 tcttgagtac atttcactta ttgtcctttt acttccccta ataatcaact tcctctgtgt 180
 taagccccaa aacaacctcc cacctgggtcc aacagcagtc ccaatttttg gtaattggct 240
 tcaagttggc aatgacttga accatcaact ccttgccacc atgtcacaaa cctatgggtcc 300
 tatattttta ctcaaaactg gttcaaaaaa cctagctgtg gtatcgaacc ctgagctagc 360
 taaccaagtt ctacacacgc aaggggtcga gtttgggtcc cgtccacgta acgttgtctt 420
 tgatatattt actggtaatg gacaagacat ggtgttcacc atttatggtg accattggcg 480
 aaaaatgagg cgtattatga cgcttccatt tttcactaac aaagtgggtc accaatatag 540
 tgatatgtgg gagaatgaga tggacctagt tgttgatgac ttgaagaaga atgaaaaagt 600
 gaaatgatgac ggaattgtga ttaggaaacg attgcagctg atgctatata acattatgta 660
 tcgaatgatg tttgatgcca agtttgagtc ccaagatgat cctttgttca ttgaggcaac 720
 aaagttaat tcagagagaa gcagattagc tcagagcttt gactacaatt atggtgattt 780
 tatccctttg cttagaccat tcttgaaagg gtaccttaac aagtgcгааag acttacaаac 840
 aaggagactt gcattcttca acaattattt tgtagggaaa agaaggaaaa taatgggtga 900
 aaatggagaa aacacaaga tatgttgtgc tattgatcac attatagatg ctgaaatgaa 960
 aggagaaata agtgagcaaa atgtactcta tatttgggag aatatcaatg ttgcagcaat 1020
 tgaacaact ctatggtcca tggaaatgggc catagctgag cttgtaaаc atcccattgt 1080
 tcaacagaag attagggatg aaatctcaac agtcctcaaa ggaaagtсag tcaaagaatc 1140
 aacctacat gagctgcctt acttgctagc aacagtaaat gaaacattaa gactccacac 1200
 accaatacct ttacttgtac cacatatgaa ccttgaagaa gcaaagctag gtgggttacac 1260
 tattcctaaa gaaactaagg tggttgtgaa tgcatgggtg ctggctaaca accctgcctg 1320
 gtggaaaaac cagaacgaat tccgcccga gcggtttctc gaggaggata gtagcacaga 1380
 ggcagctggt gctggtgcca aggttgattt caggtacttg cccttcggta tggggaggcg 1440
 gagctgcccc ggaatcatcc ttgcactgcc aattctgggg cttgtcatag ccaaactggt 1500
 gtcaaatттt gaaatgcagg ctccctcagg tgtaggaaaa gttgatacaa gtgagaаagg 1560

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agggcagttt agcttgcaaca ttgcaaaaca ttccacgggt gtcttcaagc ctattgctgc 1620
ataatattac agtttttgggt actctataaaa gatttcaatg aatattacag tttttgtaa 1680
aaaaaaaaa aaa 1693

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

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<400> SEQUENCE: 9

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Met Tyr His Leu Leu Ser Pro Ile Glu Ala Ile Val Gly Leu Val Thr
1           5           10           15
Phe Ala Phe Leu Leu Tyr Leu Leu Trp Thr Lys Lys Gln Ser Lys Ile
20           25           30
Leu Asn Pro Leu Pro Pro Lys Ile Pro Gly Gly Trp Pro Val Ile Gly
35           40           45
His Leu Phe Tyr Phe Asn Asn Asn Gly Asp 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 Lys Asn Arg Lys Leu Val Asn Gln
130          135          140
Glu Leu Leu Ser Val Ser Arg Leu Glu Lys Phe Lys His Val Arg Phe
145          150          155          160
Ser Ile Val Gln Lys Asn Ile Lys Gln 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 Gln 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 Gln Thr Phe Lys Asp Ile Asp
245          250          255
Asn Ile Ile Gln Gly Trp Leu Asp Glu His Ile Lys Lys Arg Glu Thr
260          265          270
Lys Asp Val Gly Gly Glu Asn Glu Gln 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
Leu Ala Leu His Ile Lys Trp Val Met Ala Leu Met Ile Asn Asn Lys
325          330          335
Asn Val Met Lys Lys Ala Gln Glu Glu Met Asp Thr Ile Val Gly Arg
340          345          350

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Asp Arg Trp Val Glu Glu Asn Asp Ile Lys Asn Leu Val Tyr Leu Gln
 355 360 365
 Ala Ile Val Lys Glu Val Leu Arg Leu His Pro Pro Ala Pro Leu Ser
 370 375 380
 Val Gln His Leu Ser Val Lys Asp Cys Val Val Asn Gly Tyr His Ile
 385 390 395 400
 Pro Lys Gly Thr Ala Leu Leu Thr Asn Ile Met Lys Leu Gln Arg Asp
 405 410 415
 Pro Gln Ile Trp Val Asp Pro Asp Thr Phe Asp Pro Glu Arg Phe Leu
 420 425 430
 Thr Thr Asn Ala Ala Ile Asp Tyr Arg Gly Gln His Tyr Glu Leu Ile
 435 440 445
 Pro Phe Gly Ser Gly Arg Arg Ala Cys Pro Ala Met Asn Tyr Ser Leu
 450 455 460
 Gln Val Glu His Leu Ser Ile Ala His Leu Ile Gln Gly Phe Asn Phe
 465 470 475 480
 Ala Thr Thr Thr Asn Glu Pro Leu Asp Met Lys Gln Gly Val Gly Leu
 485 490 495
 Thr Leu Pro Lys Lys Thr Asp Val Glu Val Leu Ile Thr Pro Arg Leu
 500 505 510
 Pro Pro Thr Leu Tyr Gln Tyr
 515

<210> SEQ ID NO 10

<211> LENGTH: 1578

<212> TYPE: DNA

<213> ORGANISM: Nicotiana tabacum

<400> SEQUENCE: 10

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atgtatcatc ttctttctcc catagaagcc attgtaggac ttgtaacctt tgcatttcta    60
ctctacttgc tatggacaaa aaaacaatca aaaatcctaa acccaactgcc tccaaaaatc    120
ccaggtggat ggccagtaat cggccatctc ttttatttca acaacaatgg cgatgatgac    180
cgccattttt ctcaaaaaact cgggagactta gctgacaaat atggtcccgt cttcacattc    240
cggttagggt ttcgcccgtt cttggcgggtg agtagttatg aagctatgaa agaatgcttc    300
tctaccaatg atatccattt cgccgatcgg ccagctttac tttacggaga atacctttgc    360
tataacaatg ccatgcttgc tgttgccaaa tatggccctt actggaaaaa aaatcgaaag    420
ctagtcaatc aagaacttct ctccgttagt cggctcgaaa aattcaaaaca tgttagattt    480
tctatagttc agaaaaatat taaacaattg tataattgtg attcaccaat ggtgaagata    540
aaccttagtg attggataga taaattgaca ttcgacatca ttttgaaaat ggttgttggg    600
aagacctata ataatggaca tggagaaata ctcaaagcag cttttcagaa gttcatgggt    660
caagctatgg agattgagct ctatgatgtt tttcacattc catttttcaa gtggttggat    720
cttacagggg atattaaggc tatgaaacaa actttcaaag acattgataa tattatccaa    780
ggttggttag atgagcacat taagaagaga gaaacaaagg atgttggagg tgaanaatgaa    840
caagatttta ttgatgtgct gctttccaag aggagcaacg aacatcttgg cgatggttac    900
tctcatgaca ccacatcaa agcaacagta ttcactttgg tcttggatgc aacagacaca    960
cttgcacttc atataaagtg ggtaatggcg ttaatgataa acaataagaa tgtcatgaag   1020
aaagcacaag aagagatgga caccattggt ggtagagata gatgggtaga agagaatgat   1080
atcaagaatt tgggtgatct tcaagcaatt gttaaagaag tattacgatt acatccacct   1140

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gcacctttgt cagtacaaca cctatccgta aaagattgtg ttgtcaatgg ataccatatt 1200
cctaagggga ctgcactact tacaatat atgaaacttc aacgagaccc acaaatatgg 1260
gtagatcctg atacattcga tccagaaaga ttcttgacga ctaatgctgc aattgactat 1320
cgcgggcagc actatgagtt gatcccgttt ggatcagggg gacgagcttg tccgcgatg 1380
aattactcat tgcaagtgga acacctttca attgctcatt tgatccaggg tttcaatatt 1440
gcaactacga ctaacgagcc tttggatatg aaacaaggcg tgggtctaac tttacctaag 1500
aagacagatg ttgaagtgct aattacacct cgccttcctc ctacgcttta tcaatattaa 1560
tatgttttgt tgtttgta 1578

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<210> SEQ ID NO 11
<211> LENGTH: 509
<212> TYPE: PRT
<213> ORGANISM: Nicotiana tabacum

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<400> SEQUENCE: 11

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

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Gly Gly Thr Glu Ser Ser Ala Val Thr Val Glu Trp Ala Ile Ser Glu
 305 310 315 320
 Leu Leu Lys Lys Pro Glu Ile Phe Lys Lys Ala Thr Glu Glu Leu Asp
 325 330 335
 Arg Val Ile Gly Gln Asn Arg Trp Val Gln 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 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 Glu 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 Gln 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
 465 470 475 480
 Asp Glu Ile Phe Gly Leu Ser Thr Pro Lys Lys Phe Pro Leu Ala Thr
 485 490 495
 Val Ile Glu Pro Arg Leu Ser Pro Lys Leu Tyr Ser Val
 500 505

<210> SEQ ID NO 12

<211> LENGTH: 1530

<212> TYPE: DNA

<213> ORGANISM: Nicotiana tabacum

<400> SEQUENCE: 12

atggaaggta caaacttgac tacatatgca gcagtatttc ttggtactct gtttcttttg 60
 ctcttttcca aatttctctg ccaaagaaaa ctcaacttac ctccaggccc aaaaccatgg 120
 ccgatcatcg gaaacttaaa ccttatcggc aatcttcctc atcgctcaat ccacgaactt 180
 tcaactcaagt acgggccaat tatgcaactc caattcgga ctttccccgt tgtgtgtggc 240
 tcttccgtcg aaatggccaa ggttttctc aaatcaatgg atattaactt tgtaggcagg 300
 cctaaaacgg ccgccgggaa gtacacaact tacaattatt cagatattac atggtctcct 360
 tatggaccat attggcgcca ggcacgtaga atgtgcctaa tggattatt cagcacgaaa 420
 cgtctcgatt catacagta tattcgggct gaggagttgc attctctgct ccataatttg 480
 aataaaatat cagggaaacc aattgtgctg aaagattatt tgacgacgtt gaggttaaat 540
 gttattagca ggatggtact ggggaagagg tatttgacg aatccgagaa ctcgattgtg 600
 actcctgagg aatttaagaa gatgttgac gagctgttct tgctaaatgg tgtacttaat 660
 attggagatt caattccctg gattgattc atggacttac aaggttatgt taagaggatg 720
 aaattgtga gcaagaaatt cgacaagttt ttggagcatg ttatcgatga gcataacgtt 780
 aggagaaatg gaggggagaa ttacattgct aaggacatgg ttgatgttct gttgcagctt 840
 gctgatgatc cgacgttgga agttaagctg gagagacatg gactcaaagc attcaactcag 900
 gatatgcttg ctggtggaac cgagagttca gcagtgcag tggagtgggc aatttcggag 960

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ctgctaaaga agccagagat tttcaaaaag gctacagaag aattggatcg agtaattggg 1020
cagaatagat gggtagaaga aaaagacatt ccaaatcttc cttacataga ggcaatagtc 1080
aaagagacta tgcgactgca ccccgtagca ccaatggttg tgccacggga gtgtcgagaa 1140
gactgtaagg tagcaggcta cgacgttaag aaaggaacca gggtccttgt gagcgtatgg 1200
actattggaa gagaccctac attgtgggac gagcctgagg cgttcaagcc ggagaggttc 1260
cacgaaaagt ccattgatgt taaaggacat gattttgagc ttttgccatt tggagctggg 1320
agaaggatgt gcccgggtta taacttgggg ctttaagggtga ttcaagctag cttagctaatt 1380
cttatacatg gatttaactg gtcattgctt gataatatga ctctgagga cctcgacatg 1440
gatgagatgt ttgggctctc cacacctaaa aagtttccac ttgctactgt gattgagcca 1500
agactttcac caaaacttta ctctgtttga 1530

```

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<210> SEQ ID NO 13
<211> LENGTH: 36
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Derived from Nicotiana tabacum p450 gene

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<400> SEQUENCE: 13

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```

ggcggagaat ttgtcctgga atgtcatttg gtttag 36

```

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<210> SEQ ID NO 14
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Derived from Nicotiana tabacum p450 gene

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<400> SEQUENCE: 14

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gtacaatagt gaggttgaca atg 23

```

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<210> SEQ ID NO 15
<211> LENGTH: 18
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Derived from Nicotiana tabacum p450 gene

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<400> SEQUENCE: 15

```

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ggtggttgty aatgcatg 18

```

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<210> SEQ ID NO 16
<211> LENGTH: 25
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Derived from Nicotiana tabacum p450 gene

```

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<400> SEQUENCE: 16

```

```

ttatgcagca ataggcttga agaca 25

```

```

<210> SEQ ID NO 17
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Derived from Nicotiana tabacum p450 gene

```

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<400> SEQUENCE: 17

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```

gggggatcca tgcaattctt cagcttggtt tcc 33

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<210> SEQ ID NO 18
 <211> LENGTH: 33
 <212> TYPE: DNA
 <213> ORGANISM: Artificial sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Derived from Nicotiana tabacum p450 gene

 <400> SEQUENCE: 18
 ggggaattct tactctcgag aaggtgata agg 33

<210> SEQ ID NO 19
 <211> LENGTH: 30
 <212> TYPE: DNA
 <213> ORGANISM: Artificial sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Derived from Nicotiana tabacum p450 gene

 <400> SEQUENCE: 19
 cccggatcca tgtatcatct tctttctccc 30

<210> SEQ ID NO 20
 <211> LENGTH: 33
 <212> TYPE: DNA
 <213> ORGANISM: Artificial sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Derived from Nicotiana tabacum p450 gene

 <400> SEQUENCE: 20
 ggggaattct caatattgat aaagcgtagg agg 33

<210> SEQ ID NO 21
 <211> LENGTH: 33
 <212> TYPE: DNA
 <213> ORGANISM: Artificial sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Derived from Nicotiana tabacum p450 gene

 <400> SEQUENCE: 21
 cccggatcca tgcaatcctt cagcttggtt tcc 33

<210> SEQ ID NO 22
 <211> LENGTH: 33
 <212> TYPE: DNA
 <213> ORGANISM: Artificial sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Derived from Nicotiana tabacum p450 gene

 <400> SEQUENCE: 22
 ggggagctct cactcgcaag aagattgata agg 33

<210> SEQ ID NO 23
 <211> LENGTH: 75
 <212> TYPE: DNA
 <213> ORGANISM: Artificial sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Derived from Nicotiana tabacum p450 gene

 <400> SEQUENCE: 23
 gccattatcg gcgcaatact aatctccaaa ctccgcggtg aaaaattcaa gctcccacct 60
 ggtccaacag cagtc 75

<210> SEQ ID NO 24
 <211> LENGTH: 75
 <212> TYPE: DNA
 <213> ORGANISM: Artificial sequence
 <220> FEATURE:

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<223> OTHER INFORMATION: Derived from Nicotiana tabacum p450 gene

<400> SEQUENCE: 24

gggggatcca tggacctct cctcatagaa aaaaccctcg tcgccttatt cgccgccatt 60

atcggcgcaa tacta 75

<210> SEQ ID NO 25

<211> LENGTH: 33

<212> TYPE: DNA

<213> ORGANISM: Artificial sequence

<220> FEATURE:

<223> OTHER INFORMATION: Derived from Nicotiana tabacum p450 gene

<400> SEQUENCE: 25

ggggagctct tatgcagcaa taggcttgaa gac 33

<210> SEQ ID NO 26

<211> LENGTH: 7

<212> TYPE: PRT

<213> ORGANISM: Nicotiana tabacum p450 protein

<220> FEATURE:

<221> NAME/KEY: Variant

<222> LOCATION: (7)..(7)

<223> OTHER INFORMATION: Xaa = any amino acid

<400> SEQUENCE: 26

Lys Glu Thr Leu Arg Leu Xaa
1 5

<210> SEQ ID NO 27

<211> LENGTH: 11

<212> TYPE: PRT

<213> ORGANISM: Nicotiana tabacum p450 protein

<220> FEATURE:

<221> NAME/KEY: Variant

<222> LOCATION: (4)..(4)

<223> OTHER INFORMATION: Xaa = any amino acid

<220> FEATURE:

<221> NAME/KEY: Variant

<222> LOCATION: (8)..(8)

<223> OTHER INFORMATION: Xaa = any amino acid

<400> SEQUENCE: 27

Pro Phe Gly Xaa Gly Arg Arg Xaa Cys Pro Ala
1 5 10

<210> SEQ ID NO 28

<211> LENGTH: 11

<212> TYPE: PRT

<213> ORGANISM: Nicotiana tabacum p450 protein

<220> FEATURE:

<221> NAME/KEY: Variant

<222> LOCATION: (4)..(4)

<223> OTHER INFORMATION: Xaa = any amino acid

<220> FEATURE:

<221> NAME/KEY: Variant

<222> LOCATION: (8)..(8)

<223> OTHER INFORMATION: Xaa = any amino acid

<400> SEQUENCE: 28

Pro Phe Gly Xaa Gly Arg Arg Xaa Cys Pro Gly
1 5 10

<210> SEQ ID NO 29

<211> LENGTH: 6

<212> TYPE: PRT

<213> ORGANISM: Nicotiana tabacum p450 protein

<220> FEATURE:

<221> NAME/KEY: Variant

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<222> LOCATION: (2)..(2)
<223> OTHER INFORMATION: Xaa = any amino acid

<400> SEQUENCE: 29

Phe Xaa Pro Glu Arg Phe
1           5

<210> SEQ ID NO 30
<211> LENGTH: 20
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Derived from Nicotiana tabacum p450 protein

<400> SEQUENCE: 30

Ala Ala Arg Gly Ala Arg Ala Cys Ile Tyr Thr Ile Met Gly Ile Tyr
1           5           10           15

Thr Ile Cys Ala
           20

<210> SEQ ID NO 31
<211> LENGTH: 20
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Derived from Nicotiana tabacum p450 protein

<400> SEQUENCE: 31

Ala Ala Arg Gly Ala Arg Ala Cys Ile Tyr Thr Ile Met Gly Ile Tyr
1           5           10           15

Thr Ile Thr Ala
           20

<210> SEQ ID NO 32
<211> LENGTH: 20
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Derived from Nicotiana tabacum p450 protein

<400> SEQUENCE: 32

Ala Ala Arg Gly Ala Arg Ala Cys Ile Tyr Thr Ile Met Gly Ile Tyr
1           5           10           15

Thr Ile Met Gly
           20

<210> SEQ ID NO 33
<211> LENGTH: 18
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Derived from Nicotiana tabacum p450 protein

<400> SEQUENCE: 33

Thr Thr Tyr Ile Ile Ile Cys Cys Ile Gly Ala Arg Met Gly Ile Thr
1           5           10           15

Thr Tyr

<210> SEQ ID NO 34
<211> LENGTH: 18
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Derived from Nicotiana tabacum p450 protein

<400> SEQUENCE: 34

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Arg Ala Ala Ile Cys Lys Tyr Thr Cys Ile Gly Gly Ile Ile Ile Arg
 1 5 10 15

Ala Ala

<210> SEQ ID NO 35
 <211> LENGTH: 20
 <212> TYPE: PRT
 <213> ORGANISM: Artificial sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Derived from Nicotiana tabacum p450 protein

<400> SEQUENCE: 35

Gly Gly Ile Met Gly Ile Met Gly Ile Ile Ile Ile Thr Gly Tyr Cys
 1 5 10 15

Cys Ile Gly Ser
 20

<210> SEQ ID NO 36
 <211> LENGTH: 20
 <212> TYPE: PRT
 <213> ORGANISM: Artificial sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Derived from Nicotiana tabacum p450 protein

<400> SEQUENCE: 36

Cys Lys Ile Cys Lys Ile Cys Cys Ile Ile Ile Ile Cys Cys Arg Ala
 1 5 10 15

Ala Ile Gly Gly
 20

<210> SEQ ID NO 37
 <211> LENGTH: 21
 <212> TYPE: DNA
 <213> ORGANISM: Artificial sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Derived from T7 bacteriophage promoter

<400> SEQUENCE: 37

gtaatacgac tcactatagg g 21

<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

caattaaccc tcactaaagg g 21

<210> SEQ ID NO 39
 <211> LENGTH: 500
 <212> TYPE: PRT
 <213> ORGANISM: Mentha piperita

<400> SEQUENCE: 39

Met Glu Leu Gln Ile Ser Ser Ala Ile Ile Ile Leu Val Val Thr Tyr
 1 5 10 15

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
 35 40 45

His Leu Leu Trp Gly Lys Leu Pro Gln His Ala Leu Ala Ser Val Ala

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50					55					60					
Lys	Gln	Tyr	Gly	Pro	Val	Ala	His	Val	Gln	Leu	Gly	Glu	Val	Phe	Ser
65					70					75					80
Val	Val	Leu	Ser	Ser	Arg	Glu	Ala	Thr	Lys	Glu	Ala	Met	Lys	Leu	Val
			85						90					95	
Asp	Pro	Ala	Cys	Ala	Asp	Arg	Phe	Glu	Ser	Ile	Gly	Thr	Lys	Ile	Met
			100					105					110		
Trp	Tyr	Asp	Asn	Asp	Asp	Ile	Ile	Phe	Ser	Pro	Tyr	Ser	Val	His	Trp
		115				120						125			
Arg	Gln	Met	Arg	Lys	Ile	Cys	Val	Ser	Glu	Leu	Leu	Ser	Ala	Arg	Asn
	130					135					140				
Val	Arg	Ser	Phe	Gly	Phe	Ile	Arg	Gln	Asp	Glu	Val	Ser	Arg	Leu	Leu
	145					150					155				160
Gly	His	Leu	Arg	Ser	Ser	Ala	Ala	Ala	Gly	Glu	Ala	Val	Asp	Leu	Thr
				165					170					175	
Glu	Arg	Ile	Ala	Thr	Leu	Thr	Cys	Ser	Ile	Ile	Cys	Arg	Ala	Ala	Phe
			180					185					190		
Gly	Ser	Val	Ile	Arg	Asp	His	Glu	Glu	Leu	Val	Glu	Leu	Val	Lys	Asp
		195				200					205				
Ala	Leu	Ser	Met	Ala	Ser	Gly	Phe	Glu	Leu	Ala	Asp	Met	Phe	Pro	Ser
	210					215					220				
Ser	Lys	Leu	Leu	Asn	Leu	Cys	Trp	Asn	Lys	Ser	Lys	Leu	Trp	Arg	
	225			230					235					240	
Met	Arg	Arg	Arg	Val	Asp	Ala	Ile	Leu	Glu	Ala	Ile	Val	Glu	Glu	His
				245					250					255	
Lys	Leu	Lys	Lys	Ser	Gly	Glu	Phe	Gly	Gly	Glu	Asp	Ile	Ile	Asp	Val
			260					265					270		
Leu	Phe	Arg	Met	Gln	Lys	Asp	Ser	Gln	Ile	Lys	Val	Pro	Ile	Thr	Thr
		275					280					285			
Asn	Ala	Ile	Lys	Ala	Phe	Ile	Phe	Asp	Thr	Phe	Ser	Ala	Gly	Thr	Glu
	290					295					300				
Thr	Ser	Ser	Thr	Thr	Thr	Leu	Trp	Val	Met	Ala	Glu	Leu	Met	Arg	Asn
	305					310					315				320
Pro	Glu	Val	Met	Ala	Lys	Ala	Gln	Ala	Glu	Val	Arg	Ala	Ala	Leu	Lys
				325					330					335	
Gly	Lys	Thr	Asp	Trp	Asp	Val	Asp	Asp	Val	Gln	Glu	Leu	Lys	Tyr	Met
			340					345					350		
Lys	Ser	Val	Val	Lys	Glu	Thr	Met	Arg	Met	His	Pro	Pro	Ile	Pro	Leu
		355					360					365			
Ile	Pro	Arg	Ser	Cys	Arg	Glu	Glu	Cys	Glu	Val	Asn	Gly	Tyr	Thr	Ile
	370					375					380				
Pro	Asn	Lys	Ala	Arg	Ile	Met	Ile	Asn	Val	Trp	Ser	Met	Gly	Arg	Asn
	385			390							395				400
Pro	Leu	Tyr	Trp	Glu	Lys	Pro	Glu	Thr	Phe	Trp	Pro	Glu	Arg	Phe	Asp
				405					410					415	
Gln	Val	Ser	Arg	Asp	Phe	Met	Gly	Asn	Asp	Phe	Glu	Phe	Ile	Pro	Phe
			420					425					430		
Gly	Ala	Gly	Arg	Arg	Ile	Cys	Pro	Gly	Leu	Asn	Phe	Gly	Leu	Ala	Asn
		435					440					445			
Val	Glu	Val	Pro	Leu	Ala	Gln	Leu	Leu	Tyr	His	Phe	Asp	Trp	Lys	Leu
	450					455					460				
Ala	Glu	Gly	Met	Asn	Pro	Ser	Asp	Met	Asp	Met	Ser	Glu	Ala	Glu	Gly
	465			470							475				480

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Leu Thr Gly Ile Arg Lys Asn Asn Leu Leu Leu Val Pro Thr Pro Tyr
 485 490 495

Asp Pro Ser Ser
 500

<210> SEQ ID NO 40

<211> LENGTH: 496

<212> TYPE: PRT

<213> ORGANISM: *Mentha spicata*

<400> SEQUENCE: 40

Met Glu Leu Asp Leu Leu Ser Ala Ile Ile Ile Leu Val Ala Thr Tyr
 1 5 10 15

Ile Val Ser Leu Leu Ile Asn Gln Trp Arg Lys Ser Lys Ser Gln Gln
 20 25 30

Asn Leu Pro Pro Ser Pro Pro Lys Leu Pro Val Ile Gly His Leu His
 35 40 45

Phe Leu Trp Gly Gly Leu Pro Gln His Val Phe Arg Ser Ile Ala Gln
 50 55 60

Lys Tyr Gly Pro Val Ala His Val Gln Leu Gly Glu Val Tyr Ser Val
 65 70 75 80

Val Leu Ser Ser Ala Glu Ala Ala Lys Gln Ala Met Lys Val Leu Asp
 85 90 95

Pro Asn Phe Ala Asp Arg Phe Asp Gly Ile Gly Ser Arg Thr Met Trp
 100 105 110

Tyr Asp Lys Asp Asp Ile Ile Phe Ser Pro Tyr Asn Asp His Trp Arg
 115 120 125

Gln Met Arg Arg Ile Cys Val Thr Glu Leu Leu Ser Pro Lys Asn Val
 130 135 140

Arg Ser Phe Gly Tyr Ile Arg Gln Glu Glu Ile Glu Arg Leu Ile Arg
 145 150 155 160

Leu Leu Gly Ser Ser Gly Gly Ala Pro Val Asp Val Thr Glu Glu Val
 165 170 175

Ser Lys Met Ser Cys Val Val Val Cys Arg Ala Ala Phe Gly Ser Val
 180 185 190

Leu Lys Asp Gln Gly Ser Leu Ala Glu Leu Val Lys Glu Ser Leu Ala
 195 200 205

Leu Ala Ser Gly Phe Glu Leu Ala Asp Leu Tyr Pro Ser Ser Trp Leu
 210 215 220

Leu Asn Leu Leu Ser Leu Asn Lys Tyr Arg Leu Gln Arg Met Arg Arg
 225 230 235 240

Arg Leu Asp His Ile Leu Asp Gly Phe Leu Glu Glu His Arg Glu Lys
 245 250 255

Lys Ser Gly Glu Phe Gly Gly Glu Asp Ile Val Asp Val Leu Phe Arg
 260 265 270

Met Gln Lys Gly Ser Asp Ile Lys Ile Pro Ile Thr Ser Asn Cys Ile
 275 280 285

Lys Gly Phe Ile Phe Asp Thr Phe Ser Ala Gly Ala Glu Thr Ser Ser
 290 295 300

Thr Thr Ile Ser Trp Ala Leu Ser Glu Leu Met Arg Asn Pro Ala Lys
 305 310 315 320

Met Ala Lys Val Gln Ala Glu Val Arg Glu Ala Leu Lys Gly Lys Thr
 325 330 335

Val Val Asp Leu Ser Glu Val Gln Glu Leu Lys Tyr Leu Arg Ser Val
 340 345 350

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Leu Lys Glu Thr Leu Arg Leu His Pro Pro Phe Pro Leu Ile Pro Arg
 355 360 365
 Gln Ser Arg Glu Glu Cys Glu Val Asn Gly Tyr Thr Ile Pro Ala Lys
 370 375 380
 Thr Arg Ile Phe Ile Asn Val Trp Ala Ile Gly Arg Asp Pro Gln Tyr
 385 390 395 400
 Trp Glu Asp Pro Asp Thr Phe Arg Pro Glu Arg Phe Asp Glu Val Ser
 405 410 415
 Arg Asp Phe Met Gly Asn Asp Phe Glu Phe Ile Pro Phe Gly Ala Gly
 420 425 430
 Arg Arg Ile Cys Pro Gly Leu His Phe Gly Leu Ala Asn Val Glu Ile
 435 440 445
 Pro Leu Ala Gln Leu Leu Tyr His Phe Asp Trp Lys Leu Pro Gln Gly
 450 455 460
 Met Thr Asp Ala Asp Leu Asp Met Thr Glu Thr Pro Gly Leu Ser Gly
 465 470 475 480
 Pro Lys Lys Lys Asn Val Cys Leu Val Pro Thr Leu Tyr Lys Ser Pro
 485 490 495

<210> SEQ ID NO 41
 <211> LENGTH: 509
 <212> TYPE: PRT
 <213> ORGANISM: *Nepeta racemosa*

<400> SEQUENCE: 41

Met Val Ser Leu Ser Tyr Phe Leu Ile Ala Leu Leu Cys Thr Leu Pro
 1 5 10 15
 Phe Leu Leu Phe Leu Asn Lys Trp Arg Arg Ser Tyr Ser Gly Lys Thr
 20 25 30
 Pro Pro Pro Ser Pro Pro Lys Leu Pro Val Ile Gly Asn Leu His Gln
 35 40 45
 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
 Ser Ser Pro Glu Ala Ala Arg Glu Ile Met Lys Asn Gln Asp Ile Val
 85 90 95
 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 Gln Tyr Gly Glu Tyr Trp Arg Gln Ile
 115 120 125
 Arg Ser Ile Cys Val Leu Gln Leu Leu Ser Asn Lys Arg Val Gln Ser
 130 135 140
 Phe Arg Arg Val Arg Glu Glu Glu Thr Ser Ile Met Val Glu Lys Ile
 145 150 155 160
 Met Gln Leu Gly Ser Ser Ser Ser Thr Pro Val Asn Leu Ser Glu Leu
 165 170 175
 Leu Leu Ser Leu Thr Asn Asp Val Val Cys Arg Val Thr Leu Gly Lys
 180 185 190
 Lys Tyr Gly Gly Gly Asn Gly Ser Glu Glu Val Asp Lys Leu Lys Glu
 195 200 205
 Met Leu Thr Glu Ile Gln Asn Leu Met Gly Ile Ser Pro Val Trp Glu
 210 215 220
 Phe Ile Pro Trp Leu Asn Trp Thr Arg Arg Phe Asp Gly Val Asp Gln
 225 230 235 240

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Arg Val Asp Arg Ile Val Lys Ala Phe Asp Gly Phe Leu Glu Ser Val
 245 250 255
 Ile Gln Glu His Lys Glu Arg Asp Gly Asp Lys Asp Gly Asp Gly Asp
 260 265 270
 Gly Ala Leu Asp Phe Val Asp Ile Leu Leu Gln Phe Gln Arg Glu Asn
 275 280 285
 Lys Asn Arg Ser Pro Val Glu Asp Asp Thr Val Lys Ala Leu Ile Leu
 290 295 300
 Asp Met Phe Val Ala Gly Thr Asp Thr Thr Ala Thr Ala Leu Glu Trp
 305 310 315 320
 Ala Val Ala Glu Leu Ile Lys Asn Pro Arg Ala Met Lys Arg Leu Gln
 325 330 335
 Asn Glu Val Arg Glu Val Ala Gly Ser Lys Ala Glu Ile Glu Glu Glu
 340 345 350
 Asp Leu Glu Lys Met Pro Tyr Leu Lys Ala Ser Ile Lys Glu Ser Leu
 355 360 365
 Arg Leu His Val Pro Val Val Leu Leu Val Pro Arg Glu Ser Thr Arg
 370 375 380
 Asp Thr Asn Val Leu Gly Tyr Asp Ile Ala Ser Gly Thr Arg Val Leu
 385 390 395 400
 Ile Asn Ala Trp Ala Ile Ala Arg Asp Pro Ser Val Trp Glu Asn Pro
 405 410 415
 Glu Glu Phe Leu Pro Glu Arg Phe Leu Asp Ser Ser Ile Asp Tyr Lys
 420 425 430
 Gly Leu His Phe Glu Leu Leu Pro Phe Gly Ala Gly Arg Arg Gly Cys
 435 440 445
 Pro Gly Ala Thr Phe Ala Val Ala Ile Asp Glu Leu Ala Leu Ala Lys
 450 455 460
 Leu Val His Lys Phe Asp Phe Gly Leu Pro Asn Gly Ala Arg Met Glu
 465 470 475 480
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 485 490 495
 Pro Leu Leu Leu Leu Pro Ile Pro His His Ala Ala Pro
 500 505

<210> SEQ ID NO 42

<211> LENGTH: 471

<212> TYPE: PRT

<213> ORGANISM: *Persea americana*

<400> SEQUENCE: 42

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 1 5 10 15
 Phe Leu Leu Lys Leu Asn Glu Lys Arg Glu Lys Lys Pro Asn Leu Pro
 20 25 30
 Pro Ser Pro Pro Asn Leu Pro Ile Ile Gly Asn Leu His Gln Leu Gly
 35 40 45
 Asn Leu Pro His Arg Ser Leu Arg Ser Leu Ala Asn Glu Leu Gly Pro
 50 55 60
 Leu Ile Leu Leu His Leu Gly His Ile Pro Thr Leu Ile Val Ser Thr
 65 70 75 80
 Ala Glu Ile Ala Glu Glu Ile Leu Lys Thr His Asp Leu Ile Phe Ala
 85 90 95
 Ser Arg Pro Ser Thr Thr Ala Ala Arg Arg Ile Phe Tyr Asp Cys Thr
 100 105 110

-continued

Asp Val Ala Phe Ser Pro Tyr Gly Glu Tyr Trp Arg Gln Val Arg Lys
 115 120 125
 Ile Cys Val Leu Glu Leu Leu Ser Ile Lys Arg Val Asn Ser Tyr Arg
 130 135 140
 Ser Ile Arg Glu Glu Glu Val Gly Leu Met Met Glu Arg Ile Ser Gln
 145 150 155 160
 Ser Cys Ser Thr Gly Glu Ala Val Asn Leu Ser Glu Leu Leu Leu Leu
 165 170 175
 Leu Ser Ser Gly Thr Ile Thr Arg Val Ala Phe Gly Lys Lys Tyr Glu
 180 185 190
 Gly Glu Glu Glu Arg Lys Asn Lys Phe Ala Asp Leu Ala Thr Glu Leu
 195 200 205
 Thr Thr Leu Met Gly Ala Phe Phe Val Gly Asp Tyr Phe Pro Ser Phe
 210 215 220
 Ala Trp Val Asp Val Leu Thr Gly Met Asp Ala Arg Leu Lys Arg Asn
 225 230 235 240
 His Gly Glu Leu Asp Ala Phe Val Asp His Val Ile Asp Asp His Leu
 245 250 255
 Leu Ser Arg Lys Ala Asn Gly Ser Asp Gly Val Glu Gln Lys Asp Leu
 260 265 270
 Val Asp Val Leu Leu His Leu Gln Lys Asp Ser Ser Leu Gly Val His
 275 280 285
 Leu Asn Arg Asn Asn Leu Lys Ala Val Ile Leu Asp Met Phe Ser Gly
 290 295 300
 Gly Thr Asp Thr Thr Ala Val Thr Leu Glu Trp Ala Met Ala Glu Leu
 305 310 315 320
 Ile Lys His Pro Asp Val Met Glu Lys Ala Gln Gln Glu Val Arg Arg
 325 330 335
 Val Val Gly Lys Lys Ala Lys Val Glu Glu Glu Asp Leu His Gln Leu
 340 345 350
 His Tyr Leu Lys Leu Ile Ile Lys Glu Thr Leu Arg Leu His Pro Val
 355 360 365
 Ala Pro Leu Leu Val Pro Arg Glu Ser Thr Arg Asp Val Val Ile Arg
 370 375 380
 Gly Tyr His Ile Pro Ala Lys Thr Arg Val Phe Ile Asn Ala Trp Ala
 385 390 395 400
 Ile Gly Arg Asp Pro Lys Ser Trp Glu Asn Ala Glu Glu Phe Leu Pro
 405 410 415
 Glu Arg Phe Val Asn Asn Ser Val Asp Phe Lys Gly Gln Asp Phe Gln
 420 425 430
 Leu Ile Pro Phe Gly Ala Gly Arg Arg Gly Cys Pro Gly Ile Ala Phe
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 Asn Trp Glu Leu Pro Gly Ile
 465 470

<210> SEQ ID NO 43
 <211> LENGTH: 509
 <212> TYPE: PRT
 <213> ORGANISM: Arabidopsis thaliana

<400> SEQUENCE: 43

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-continued

Ser	Phe	Ile	Phe	Ile	Phe	Phe	Phe	Lys	Lys	Leu	Leu	Ser	Phe	Ser	Arg
			20					25					30		
Lys	Asn	Met	Ser	Glu	Val	Ser	Thr	Leu	Pro	Ser	Val	Pro	Val	Val	Pro
		35					40					45			
Gly	Phe	Pro	Val	Ile	Gly	Asn	Leu	Leu	Gln	Leu	Lys	Glu	Lys	Lys	Pro
	50					55					60				
His	Lys	Thr	Phe	Thr	Arg	Trp	Ser	Glu	Ile	Tyr	Gly	Pro	Ile	Tyr	Ser
65					70					75					80
Ile	Lys	Met	Gly	Ser	Ser	Ser	Leu	Ile	Val	Leu	Asn	Ser	Thr	Glu	Thr
				85					90					95	
Ala	Lys	Glu	Ala	Met	Val	Thr	Arg	Phe	Ser	Ser	Ile	Ser	Thr	Arg	Lys
			100					105						110	
Leu	Ser	Asn	Ala	Leu	Thr	Val	Leu	Thr	Cys	Asp	Lys	Ser	Met	Val	Ala
		115					120						125		
Thr	Ser	Asp	Tyr	Asp	Asp	Phe	His	Lys	Leu	Val	Lys	Arg	Cys	Leu	Leu
	130					135						140			
Asn	Gly	Leu	Leu	Gly	Ala	Asn	Ala	Gln	Lys	Arg	Lys	Arg	His	Tyr	Arg
145					150					155					160
Asp	Ala	Leu	Ile	Glu	Asn	Val	Ser	Ser	Lys	Leu	His	Ala	His	Ala	Arg
				165					170					175	
Asp	His	Pro	Gln	Glu	Pro	Val	Asn	Phe	Arg	Ala	Ile	Phe	Glu	His	Glu
			180					185						190	
Leu	Phe	Gly	Val	Ala	Leu	Lys	Gln	Ala	Phe	Gly	Lys	Asp	Val	Glu	Ser
		195					200					205			
Ile	Tyr	Val	Lys	Glu	Leu	Gly	Val	Thr	Leu	Ser	Lys	Asp	Glu	Ile	Phe
	210					215						220			
Lys	Val	Leu	Val	His	Asp	Met	Met	Glu	Gly	Ala	Ile	Asp	Val	Asp	Trp
225					230					235					240
Arg	Asp	Phe	Phe	Pro	Tyr	Leu	Lys	Trp	Ile	Pro	Asn	Lys	Ser	Phe	Glu
				245					250					255	
Ala	Arg	Ile	Gln	Gln	Lys	His	Lys	Arg	Arg	Leu	Ala	Val	Met	Asn	Ala
			260					265						270	
Leu	Ile	Gln	Asp	Arg	Leu	Lys	Gln	Asn	Gly	Ser	Glu	Ser	Asp	Asp	Asp
		275					280						285		
Cys	Tyr	Leu	Asn	Phe	Leu	Met	Ser	Glu	Ala	Lys	Thr	Leu	Thr	Lys	Glu
	290					295						300			
Gln	Ile	Ala	Ile	Leu	Val	Trp	Glu	Thr	Ile	Ile	Glu	Thr	Ala	Asp	Thr
305					310					315					320
Thr	Leu	Val	Thr	Thr	Glu	Trp	Ala	Ile	Tyr	Glu	Leu	Ala	Lys	His	Pro
				325					330					335	
Ser	Val	Gln	Asp	Arg	Leu	Cys	Lys	Glu	Ile	Gln	Asn	Val	Cys	Gly	Gly
			340					345						350	
Glu	Lys	Phe	Lys	Glu	Glu	Gln	Leu	Ser	Gln	Val	Pro	Tyr	Leu	Asn	Gly
		355					360						365		
Val	Phe	His	Glu	Thr	Leu	Arg	Lys	Tyr	Ser	Pro	Ala	Pro	Leu	Val	Pro
		370				375						380			
Ile	Arg	Tyr	Ala	His	Glu	Asp	Thr	Gln	Ile	Gly	Gly	Tyr	His	Val	Pro
385					390					395					400
Ala	Gly	Ser	Glu	Ile	Ala	Ile	Asn	Ile	Tyr	Gly	Cys	Asn	Met	Asp	Lys
				405					410					415	
Lys	Arg	Trp	Glu	Arg	Pro	Glu	Asp	Trp	Trp	Pro	Glu	Arg	Phe	Leu	Asp
			420					425						430	
Asp	Gly	Lys	Tyr	Glu	Thr	Ser	Asp	Leu	His	Lys	Thr	Met	Ala	Phe	Gly
		435					440						445		

-continued

Ala Gly Lys Arg Val Cys Ala Gly Ala Leu Gln Ala Ser Leu Met Ala
 450 455 460

Gly Ile Ala Ile Gly Arg Leu Val Gln Glu Phe Glu Trp Lys Leu Arg
 465 470 475 480

Asp Gly Glu Glu Glu Asn Val Asp Thr Tyr Gly Leu Thr Ser Gln Lys
 485 490 495

Leu Tyr Pro Leu Met Ala Ile Ile Asn Pro Arg Arg Ser
 500 505

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 5
P450 polypeptide is encoded by nucleic acid that can be
amplified with degenerate primers based on one of SEQ ID
NOS: 26-29.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO. : 8,445,231 B2
APPLICATION NO. : 13/199349
DATED : May 21, 2013
INVENTOR(S) : Chappell et al.

Page 1 of 2

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'-GCCATTATCGGCGCAATACTAATC
TCCAAACTCCGCGGTAAAAAATTCAAGCTCCACCTGGTCCAACAGCAGTC-
3'” with --5'-GCCATTATCGGCGCAATACTAATCTCCAAACTCCGCGGTAAAAA
TTCAAGCTCCACCTGGTCCAACAGCAGTC-3'--;

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



Michelle K. Lee
Deputy Director of the United States Patent and Trademark Office

at column 14, lines 25-27, please replace “ 5'-GGGGGATCCATGGACCTCCTCCTC
ATAGAAAAAACCCCTCGTCGCCTTATTCGCCGCCATTATCGGCGCAATACTA-
3'” with -- 5'-GGGGGATCCATGGACCTCCTCCTCATAGAAAAAACCCCTCGTCGC
CTTATTCGCCGCCATTATCGGCGCAATACTA-3'--;

at column 15, line 67, please replace “100 tI” with --100 μ l--;

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

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