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REVIEW ARTICLE

# Plant cytochrome P450s: nomenclature and involvement in natural product biosynthesis

**Saiema Rasool**[1] · **Rozi Mohamed**[1]

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**Abstract** Cytochrome P450s constitute the largest family of enzymatic proteins in plants acting on various endogenous and xenobiotic molecules. They are monooxygenases that insert one oxygen atom into inert hydrophobic molecules to make them more reactive and hydro-soluble. Besides for physiological functions, the extremely versatile cytochrome P450 biocatalysts are highly demanded in the fields of biotechnology, medicine, and phytoremediation. The nature of reactions catalyzed by P450s is irreversible, which makes these enzymes attractions in the evolution of plant metabolic pathways. P450s are prime targets in metabolic engineering approaches for improving plant defense against insects and pathogens and for production of secondary metabolites such as the anti-neoplastic drugs taxol or indole alkaloids. The emerging examples of P450 involvement in natural product synthesis in traditional medicinal plant species are becoming increasingly interesting, as they provide new alternatives to modern medicines. In view of the divergent roles of P450s, we review their classification and nomenclature, functions and evolution, role in biosynthesis of secondary metabolites, and use as tools in pharmacology.

**Keywords** Medicinal plants · Pharmacology · Terpenoids · Plant-derived drugs

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✉ Rozi Mohamed
rozimohd@upm.edu.my

[1] Forest Biotech Laboratory, Department of Forest Management, Faculty of Forestry, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia

## Introduction

Plants being green factories are sanctified by an amazing variety of low-molecular-weight natural products that play vital roles in adaptation and defense. They are called secondary metabolites or bioactive natural products. Although the general metabolites are found in all plant species, the secondary metabolites are found only in a few numbers of species. These specialized compounds can be clustered biochemically into three major groups: terpenoids, phenylpropanoids, and nitrogen-containing compounds. Further, the nitrogen-containing compounds can be sub-divided into alkaloids, cyanogenic glucosides, and glucosinolates. These compounds are essential for the species realization as their evolution leads to the development and adaptation and therefore are dynamic force in speciation, such as terpenoids that function as structural components of membranes, phytohormones, chemical defense compounds, and signaling molecules. The same way, phenylpropanoids are phytoalexins and function as UV protectants (flavonoids and sinapoly esters), trapping free radical vascularization, while also performing signaling responsibilities (Hamberger and Bak 2013; Kumar et al. 2015). A majority of these specialized metabolites are oxygenated, and the versatile catalysts like cytochrome P450s (P450s) are often recruited in the biosynthesis of plants' specialized compounds; they are the key to structural and secondary metabolite diversity in plants (Mizutani et al. 2011; Schuler 2011). Oxidative reactions including hydroxylation, epoxidation, dealkylation, dehydration, and carbon-carbon bond cleavage are catalyzed by P450s (Schuler and Werck-Reichhart 2003). P450s have been found to be involved in the biosynthesis of plant hormones; defensive secondary metabolites (like phytoalexins); a variety of compounds like phenylpropanoids, terpenoids, and fatty acids; and in detoxification of exogenous chemicals such as herbicides (Werck-Reichhart et al. 2000; Nelson 2011).

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The heme-containing P450s are one of the largest families of plant proteins involved in the formation of a large proportion of bioactive natural products (Morant et al. 2003; Nielsen and Moller 2005). In 1958, Klingenberg (1958) and Garfinkel (1958) were the first to identify P450s on the basis of their spectral properties. The P450s have been found in all organisms from bacteria to animals and plants. They are mostly bound to the cytoplasmic surface of the endoplasmic reticulum (ER) and catalyze a range of oxygenation/hydroxylation reactions that involve molecular oxygen and are reliant on electron transfer from NADPH via NADPH-P450 reductase. Almost all the eukaryotic P450s are composed of two integral membrane proteins, the P450 and the NADPH-cytochrome P450 oxido-reductase, and have been named on the basis of their cytoplasmic localization and spectrophotometric peak revealed from maximum absorption of the protein at 450 nm (Bak et al. 2011). P450s are heme-thiolate proteins having a hydrophobic pocket, a protoporphyrin-IX linked to the apoprotein by a bond between the heme iron center and the sulfur atom of a conserved cysteinyl residue. Normally, in P450-catalyzed reactions, activation occurs when molecular oxygen binds to the heme iron before being transferred to the substrate. Carbon monoxide (CO) also binds to the heme iron and forms a reduced P450, making a specific CO-binding modification spectrum with an absorbance maximum at 450 nm (Van Bogaert et al. 2011). The P450-CO complex is inactive and named P450 (Omura and Sato 1964). The characteristic feature of most of the P450-catalyzed reactions is the inhibition by CO and reversion of this inhibition by 450-nm light. Thus, P450s play a key function in generating diverse chemicals that is the hallmark of plants compared with animals.

## Nomenclature and classification

Members of the P450 gene superfamily are genetically diverse, and their sequence conservation is particularly low. In attempt to create a nomenclature system, individual genes were named and allocated into families and sub-families based on their relatedness at amino acid sequence level and phylogenetic association as decided by the P450 Nomenclature Committee (http://drnelson.utmem.edu/CytochromeP450.html). Following this nomenclature, an Arabic numeral is assigned for the family and a letter for the sub-family. Members within a sub-family are numbered in a series. Pseudogenes, which do not produce functional proteins, are assigned by the addition of the letter "P" immediately after the numeral family designation.

P450 sequences are omnipresent in all realms of living organisms. Often, the blocks of numbers have been reserved for categorizing new cytochrome P450 families under taxonomic ancestries such as CYP1-CYP49 and CYP301-CYP499 for animals, CYP51-CYP69 and CYP501-CYP699 for lower eukaryotes, CYP71-CYP99 and CYP701-CYP999 for plants, and CYP101-CYP299 for bacteria. However, these designations do not reflect the phylogenetic relatedness of individual cytochrome P450s.

All cytochrome P450s were named according to the standard CYP450 nomenclature sequences from a well-annotated CYP450 database (Nelson 2006; Nelson 2009) as reference sequences. P450s were grouped when sharing ≥40, ≥55, and 97 % sequence identities as cutoffs for family, sub-family, and allelic variants, respectively (Chen et al. 2014). For plant cytochromes, if the sequence did not match any reference P450 sequences with identity >40 %, it will be regarded as contamination from non-plant sources.

Plants hold records in the number of P450 genes per genome such as 245 in *Arabidopsis* and 343 in *Oryza sativa*; however, less than 100 P450s are present in animal genome (Nelson et al. 2011; Hamberger and Bak 2013). Vascular lycopods such as *Selaginella moellendorffii* contain 227 P450 genes, while non-vascular bryophytes contain a more limited set, for example, 71 genes in *Physcomitrella patens.* In plant genome, the number of P450s has been significantly increased through evolution, to a point in angiosperms; they represent about 1 % of the encoded genes. In the nomenclature system of plant P450s, initially families were given numbers from 71 to 99. However, in the beginning of 1996, the numbers had expanded from 701 to 772 and are continuously being added with the latest addition of *CYP805A* (Nelson 2009, http://drnelson.uthsc.edu/cytochromeP450.html) (reviewed by Hamberger and Bak 2013).

The existing families are further assembled into phylogenetically relevant higher-ordered clusters called "clans," according to the nomenclature committee. Clans have been defined as groups of P450 families that consistently cluster together in phylogenetic trees and are named according to the lowest family member (Nelson et al. 2004). On the basis of available sequences, P450s in land plants can be divided into 11 phylogenetic distinct clans (Bak et al. 2011; Nelson et al. 2004). These clans are further grouped into two clans: single-family clans (CYP51, CYP74, CYP97, CYP710, CYP711, CYP727, CYP746) and multifamily clans (CYP71, CYP72, CYP85, CYP86) (Nelson and Reichhart 2011), though new clans are expected to emerge as more plants are sequenced.

Initially, plant P450s were grouped into A-type and non-A-type clades (Durst and Nelson 1995; Kahn and Durst 2000). Most of the A-type group appeared to be involved in the biosynthesis of secondary metabolites while the non-A-type composed of different groups and function in lipid or hormone metabolism (Paquette et al. 2000). For example, the CYP71 clan now includes the A-type P450s while the remaining ten clans are non-A-type. In A-type or CYP71 clan, continuous gene duplication procedures known as "blooms" (Feyereisen 2011; Sezutsu et al. 2013) create species-specific families, of which few duplications are indicative of ancient whole-

genome duplication actions in terrestrial plants that have led to duplicated and triplicated polyploidy ancestors (Proost et al. 2011). They have untied vast chances for neo- and sub-functionalization for the evolution of new chemistries (Adam and Wendel 2005; Ober 2010). The rest of the P450s had resulted from the retro-position actions through meiosis. Gene duplication events obtained from retro-positions are intron less by nature, and P450s intron-less blooms are widespread in *Arabidopsis* CYP86 and CYP96 families in the CYP86 clan; CYP89, CYP81, and CYP98 families in the CYP71 clan, and the CYP710 clan (Bak et al. 2011).

CYP71 clan is considered the youngest clan, as they are dominant in early plants; more than half of the P450s found in mosses are members of this clan (Nelson and Werck-Reichhart 2011; Schuler 2011). CYP710 clan has only one sub-family CYP710A that mediates phytohormone brassinolide biosynthesis (Morikawa et al. 2006; Morikawa et al. 2009). Another clan, CYP711 has a single family, CYP711A, which leads to the strigolactone signaling. In the same way, CYP74 clan constitutes its own clade and comprises few numbers of sub-families involved in metabolizing oxygenated polyunsaturated C18 fatty acid hydroperoxides to oxylipids in the octadecanoid pathway producing signaling molecules like jasmonates, important for host immunity and plant development (Hughes et al. 2009). All these single-family clans have important roles like in formation of sterols for membranes, phytohormones for plant development, and plant defense. Multi-family clans like CYP71, CYP85, and CYP86 are involved in metabolism of both general and particular compounds. CYP71 is involved in a majority of specialized compounds such as hormones and biopolymers. CYP85 metabolizes terpenoid phytohormones and CYP86 in hydroxylation and epoxidation of fatty acids, fatty alcohols, or alkanes (reviewed in Hamberger and Bak 2013).

## Roles of cytochrome P450s in phytoremediation, plant defense, and pharmacology

P450 is a huge superfamily of enzymes linked to the intricate and versatile biosynthetic pathways of a wide variety of natural products that facilitate the higher plants' interaction with other organisms including plants, insects, pathogens, and herbivores. Biosynthesis of natural products like fatty acids, terpenoids, phenylpropanoids, cyanogenic glucosides, glucosinolates, and alkaloids occurs by the participation of P450 through primary and secondary metabolisms. Moreover, P450s are strictly substrate-specific enzymes, which catalyze highly regio- and stereo-selective steps in the biosynthesis of bioactive compounds (Jensen and Moller 2010; Nelson et al. 2004). These oxidation reactions are generally slow when compared to other steps in the same pathway and difficult to perform by synthetic chemistry. Most of the P450 catalytic functions are still unknown. However, the natural products that they help synthesize are marked targets in developing plants with better nutritional value or high resistance to herbivore and pest. Genetic and metabolic engineering using the P450 genes has produced transgenic plants with improved insect resistance, crops with high tolerance to herbicide, and plants for use in bioremediation. Apart from this, natural products are essential to humans with respect to aroma, condiments, flavors, nutraceuticals, and drugs. Currently, about 25 % of the available medicines constitute substances from plant origins and their secondary metabolites.

### Phytoremediation

Phytoremediation, the use of plants for the remediation of soil and ground water pollution, is an inexpensive and efficient method of remediating contaminant sites than the conventional methods of active cleanup. Plants have been shown to take up and, in some cases, to metabolize organic pollutants (Schnoor 1995), excessive nutrients (Schnoor 1995), and metals (Chaney 1997; Salt et al. 1995). Phytoremediation for some common pollutants like trichloroethylene (TCE), carbon tetrachloride, chloroform, benzene, vinyl chloride, and ethylene dibromide is possible using genetically engineered plants expressing a mammalian P450 gene (*P4502E1*). Transgenic tobacco containing the *P4502E1* gene was able to remove about 98 % of the ethylene dibromides as compared to 63 % removal by the null vector control plants (Doty et al. 2000). Genetically transformed hybrid poplars (*Populus tremula* × *Populus alba*) overexpressing *P4502E1* were capable of enhanced metabolism of TCE, vinyl chloride, carbon tetrachloride, chloroform, and benzene (Doty et al. 2007).

The use of herbicides in modern agriculture increases the yield and quality of crops, but its continuous use poses a serious concern for food safety and the environment. These harmful compounds can be retained in the soil causing pollution of underground water used for drinking purposes in many parts of the world (Pesce et al. 2011; Mench et al. 2009). Among other methods for cleaning the pollutants in the soil is through phytoremediation. P450s have been reported as key enzymes responsible for herbicide resistance in plants. They are responsible for herbicide metabolism via the phenolic or lipid pathways (Pierrel et al. 1994; Cabello-Hurtado et al. 1998). Currently, different evidences have proposed that advanced herbicide resistance in plant is due to the increased level of P450 activity (Werck-Reichhart et al. 2000; Li et al. 2012). In Asia, a population of *Phalaris minor* weeds (a phenylurea herbicide-resistant plant) was used to convert prosulfuron diclofop and chlortoluron by P450s into many metabolites (Malcolm et al. 1990). This natural phenomenon leads to the idea of genetically manipulating P450 enzymes for their application in engineering herbicide tolerance in plants. For example, the *CYP76B1* gene cloned from

*Helianthus tuberosus* (Jerusalem artichoke) was found responsible for chlortoluron resistance (Robineau et al. 1998). When expressed in yeast together with *Arabidopsis* P450 reductase, it catalyzes the dealkylation of several planar xenobiotics and phenylurea like chlortoluron (Robineau et al. 1998; Didierjean et al. 2002). The *CYP71A10* gene from soybean (Siminszky et al. 1999) and *CYP71A11* from tobacco (Yamada et al. 2000) have shown high tolerance to chlortoluron. The *CYP71C6V1* gene in wheat metabolizes the sulfonylurea herbicides like triasulfuron and chlorsulfuron (Xiang et al. 2006). Overexpression of the *CYP71B1* gene in yeast shows resistance to chlortoluron absorption (Lamb et al. 1998). When comparisons made between these P450s, only *CYP76B1*-dependent metabolism of chlortoluron is processed by N-demethylation, while the other P450s perform the ring-methyl group oxidation (Li et al. 2012). *CYP81B2* and *CYP71A11* isolated from tobacco showed chlortoluron metabolism (Yamada et al. 2000). Even though some P450s are involved in herbicide metabolism, they cannot complete an efficient catabolic pathway to detoxify herbicides and other toxic compounds (Morant et al. 2003; Yamada et al. 2000). The molecular mechanism of herbicide resistance facilitated by P450s in plants is still unclear. Nevertheless, P450 has been considered a powerful tool for phytoremediation, but it needs a perfect host plant in which the gene can be deployed to express its full potential (Macek et al. 2008).

## Enriched plant defense

P450s are involved in plant development and chemical and environmental stresses responses, via the natural products that they help to synthesize. These compounds may act directly as toxins or biocides against hostile plant species, pathogens, insects, and other herbivores (Schultz 2002) or indirectly, as stress signals to activate the plant's defense biological functions of P450s, many of which have been investigated using several strategies. In *Arabidopsis*, expression and co-expression studies have provided much evidence for the functional explanation of P450s. Biotic and abiotic stress responses using complementary DNA (cDNA) micro-array of genes in the P450 superfamily were studied by Narusaka et al. (2004). Extensive co-expression analysis tool for P450 superfamily was generated by the Wreck-Reichhart group (Ehlting et al. 2006; Ehlting et al. 2008) which provided novel signs to the functions, metabolic pathways, and regulatory networks of individual P450s. Enormous data on P450 expressions have provided information into the biological functions of P450s in plant development and the responses to chemical and environmental stresses. In *Arabidopsis*, the *CYP71A19*, *CYP71B19*, *CYP71B20*, *CYP71B26*, *CYP71B28*, *CYP76C2*, *CYP86B1*, *CYP89A9*, and *CYP94B3* are all induced when given abscisic acid (ABA) and indole-3yl-acetic acid (IAA) treatments and osmotic stress, in patterns similar to *CYP707A1* gene, which is known to mediate ABA catabolism (Schuler et al. 2006; Godiard et al. 1998). Heterologous expression has unveiled the biochemical functions of *Arabidopsis CYP735A1* and *CYP735A2* as cytokinin hydroxylases, which catalyze the biosynthesis of trans-zeatin (tZ) using an adenosine phosphate isopentenyl transferase (*AtIPT4*)/P450 co-expression system in yeast (Takei et al. 2004). In yeast, four clones of CYP707A (1–4) genes have functional ABA 8-hydroxylase activity and all of them were upregulated when given drought treatment (Kushiro et al. 2004).

Stress signaling pathways have been manipulated to increase the production of signaling molecules, which can improve plants' protection. Synchronized action of lipases and lipoxygenases produces hydroperoxy fatty acids, which act as substrates for the CYP74 family, and transforms them to oxylipins, molecules with signaling properties in plant development and defense (Noordermeer et al. 2001; Weber 2002). The four CYP74 sub-families control hydroperoxide entry into different pathways providing balancing, synergistic, and overlapping assistances to plant defense (Morant et al. 2003). The first sub-family, CYP74A, represents allene oxide synthase, which takes part in the biosynthesis of jasmonate as confirmed in a knockout mutant of *Arabidopsis thaliana* (Park et al. 2002). However, the CYP74A overexpression is insufficient to enhance the basal levels of jasmonate because of limited availability of the substrate. Although plant wounding has been found to overexpress CYP74A and leads to higher and earlier accumulation of jasmonate, unfortunately, impact on plant's defense has not been described (Park et al. 2002; Laudert et al. 2000). The second group, CYP74B, a hydroperoxide lyase, produces the wound hormone traumatin and volatile aldehyde, hexanal, and 3-hexenal, which distress the fertility of insects and help in mild wound signals (Bate and Rothstein 1998). The third group, CYP74C, catalyzes the same reactions as CYP74A and CYP74B but differs in having marked substrate specificity for 9-hydroperoxides (Matsui et al. 2000; Itoh et al. 2003). The exact role of CYP74A, CYP74B, and CYP74C in defense and plant development is yet to be understood. The last sub-family, CYP74D enzyme is a divinyl ether synthase that forms anti-microbial compounds and helps to protect the plant roots from pests (Itoh and Howe 2001).

## Plant-derived drugs

Many useful chemotherapeutic agents have been produced from plants like anti-mitotic, anti-neoplastic, anti-hypertensive, and anti-arrhythmic agents (Nielsen and Moller 2005). They possess potent biological activities that are important to the well-being of the humankind, but some have very complex structures that cannot be artificially manufactured at reasonable costs, causing humans to rely heavily on natural sources, i.e., the plant species. Typically, the compound has to be

extracted from the plant, which has very little amount of the specific compound to begin with. The extraction process often starts with a huge amount of raw material, but the yield is low, and the process is time-consuming. Therefore, conventional extraction is not a sustainable practice. To increase biological production, a comprehensive understanding of the biosynthetic pathway, the enzymes catalyzing the sequence of reactions especially the rate-limiting steps, and the genes encoding these proteins is needed. Here, we describe the role of P450s in the biosynthesis of several plant-derived drugs.

Taxol is one of the classical instances elucidating the role of P450s in its biosynthetic pathway (Walker and Croteau 2001; Jennewein and Croteau 2001). It is one of the potent anti-mitotic drugs used to treat different types of carcinomas, melanomas, and sarcomas. Diterpenoid taxol (generic name paclitaxel) is derived from the bark of the Pacific yew (*Taxus brevifolia*) in very low amounts and, thus, requires biotechnology approaches to improve its production, either through interpretation or manipulation of its biosynthetic pathway. There are about 14 *CYP* genes involved in the taxol pathway (reviewed by Morant et al. 2003); through genetic engineering, it is expected that the production of taxol and its new derivatives will be increased (Dejong et al. 2006; Lamb et al. 2007)

Terpenoid indole alkaloids (TIAs) are a large group of chemotherapeutic agents derived from different plant families like Apocynaceae, Loganiaceae, Rubiaceae, and Nyssaceae. These agents not only are structurally diverse, but also have different pharmaceutical activities like anti-neoplastic (vincristine and vinblastine), anti-hypertensives (reserpine and ajmalicine), and anti-arrhythmic (ajmaline). These pharmaceutical compounds are present in very small quantities from the respective plants. High price of the TIAs has urged researchers to dissect their biosynthetic pathways as a prerequisite if their yields were to be increased through biotechnological applications. Roles of several P450s have been characterized, such as in the hydroxylation of the bisindole alkaloid precursor tabersonin in *Catharanthus roseus* (St-Pierre and De Luca 1995) and transformation of loganin into secologanin and hydroxylation of deoxyloganin in *Lonicera japonica* (Yamamoto et al. 2000; Katano et al. 2001). P450s catalyze highly specific reactions in plants. For example, CYP71D12 acts as a tabersonin 16-hydroxylase (Schroder et al. 1999), CYP72A1 as a secologanin synthase (Irmler et al. 2000), and CYP76B6 as a geraniol/nerol 10-hydroxylase involved in the route to monoterpenoid indole alkaloids in *C. roseus* (Collu et al. 2001). For the synthesis of the terpenoid moiety secologanin and controlled production of vindoline in *C. roseus*, the *CYP76B6* and *CYP71D12* genes play key roles in the initial and rate-limiting steps, respectively (Verpoorte and Memelink 2002). Both genes yielded positive response when given elicitation or grown under certain conditions (Schroder et al. 1999; Collu et al. 2001; Contin et al. 1999). Hence, even in cell suspension cultures, they are likely the influencing factors for alkaloid production.

A large array of steroidal lactone triterpenoid called withanolides from the highly reputed medicinal plant *Withania somnifera* is an important biomolecule. Withanolides have many pharmaceutical properties like anti-oxidant, anti-inflammatory, immune modulating, and anti-stress (Bhatnagar et al. 2005; Rana et al. 2013) and possess therapeutic activities like anti-tumor and anti-bacterial (Murthy et al. 2010; Ahmad et al. 2010; Cooley et al. 2009). Various withanolides (withanolide A, withanolide D, withaferin A, and withanone) are synthesized by the hydroxylation reactions catalyzed by CYP450s from 24-methylene cholesterol, as these enzymes catalyze most of the oxidation/hydroxylation steps in plant secondary metabolism (Coon 2005; Hatlestad et al. 2012). Several CYP450s cloned from *W. somnifera* are found responsive to treatments such as methyl jasmonate, salicylic acid, wounding, light, and auxin (Srivastava et al. 2015); a few are positively correlated with the higher production of withanolides (Rana et al. 2014).

Another plant-derived drug is ginsenoside, a major triterpenoidal drug from the medicinal plant *Panax ginseng*. Ginsenosides were classified into protopanaxadiol, protopanaxatriol, and oleane types and further sub-classified into major and minor ginsenosides with respect to type/position of the sugar molecules (Choi 2008; Subramaniyam et al. 2014). Each ginsenoside has its own pharmacological activities for treating cancer, diabetes, obesity, osteoporosis, Alzheimer's, Parkinson's disease, HIV, and sexual dysfunctions and also contains other anti-oxidant elements (Hwang et al. 2012). During ginsenoside biosynthesis, protopanaxadiol synthase, a CYP enzyme (CYP716A47), catalyzes the hydroxylation of dammarenediol-II to yield protopanaxadiol. Another enzyme, hydroxylase, from the sub-family CYP716A (*CYP716A53v2*) transforms protopanaxadiol into protopanaxatriol, which is an important step in the formation of saponins in ginseng (Han et al. 2013; Villa-Ruano et al. 2013).

Triterpenoid saponins are a class of secondary metabolites used as a source of drugs found in many plant species including medicinal plants like *Medicago* (Jenner et al. 2005). Saponins in this genus are a complex mixture of triterpenic glycosides that possess a broad spectrum of biological properties such as anti-fungal, insecticidal, phytotoxic, allelopathic, and hemolytic (Tava and Avato 2006) and are also thought to participate in plant defense mechanism (Carelli et al. 2011). All of the triterpenic compounds are synthesized from the isoprenoid pathway via the cyclization of 2,3-oxidosqualene to β-amyrin, mediated by cytochrome P450 monooxygenases (Tava et al. 2010). In *Medicago truncatula*, CYP716A12 catalyzes saponin synthesis yielding oleanolic acid (Carelli et al. 2011). Another important triterpenoid saponin is glycyrrhizin from underground parts of *Glycyrrhiza* plant (Licorice).

Glycyrrhizin has many pharmacological activities like anti-inflammatory (Kroes et al. 1997), hepatoprotective (Jeong et al. 2002), anti-ulcer (He et al. 2001), anti-allergy, and anti-viral (Park et al. 2004; Fiore et al. 2008). The initial P450 enzyme in glycyrrhizin biosynthesis is β-amyrin 11-oxidase encoded by *CYP88D6* followed by *CYP72A154*, which oxidizes 11-oxo-β-amyrin into glycyrrhizin aglycone (Seki et al. 2011a).

Plant P450s have been manipulated for application in drug biosynthesis. A good example is the synthesis of artemisinin, an anti-malarial drug from a drug precursor, artemisinic acid, using a simple organism, *Saccharomyces cerevisiae* (Ro et al. 2006). By introducing specific genes encoding for amorphadiene synthase and *CYP71AV1* from *Artemisia annua* into the engineered yeast, the simple organism was able to produce artemisinic acid (Ro et al. 2006). Such approach has paved a novel method for the synthesis of drugs encoded by complex biosynthetic pathways in microorganisms like yeast and *Escherichia coli*.

## P450s in terpenoid metabolism in plants

Nearly all phases in plant development depend on P450s, as they participate via a variety of compounds throughout primary and secondary metabolisms. Such compounds are terpenoids, which form a large group of plant natural products with isoprene as their structural unit including monoterpene compounds, di-, tri-, and tertra-terpenoid compounds. Their chemical diversification has considerably extended their role in plant adaptation, protection, and the interaction of plants with their biosphere. Some terpenoids play vital role in plant growth and development, while others help in interactions with the environment. Apart from this, terpenoids are used as drugs, spices, and insecticides (Gershenzon and Dudareva 2007). The first P450s identified, characterized, and cloned from plants are those oxidizing terpenoids.

In plants, mevalonate pathway (MVA) and deoxy-xylulose-5-phosphate pathway (DXP) are the two biosynthesis pathways for terpenoids. Both the pathways co-operate and lead to the formation of various structures of terpenoids. Hence, formation of terpenoids does not involve only one step but entail special modifications including skeleton structure rearrangements. Consequently, the alteration enzymes involved in the terpenoid synthesis are reviewed in detail by Zhao et al. (2014). Biosynthesis of terpenoids in some plants in which P450 enzymes are required for the biosynthesis of bioactive products but not necessarily in the final step is tabulated in Table 1. Nowadays, the plant genetic research related to secondary metabolism genes is the most active field and has revealed hasty progress. Here, we discuss the roles of P450 genes in terpenoid biosynthesis in selected plants.

**Table 1** A list of P450s participating in the synthesis pathway of terpenoid-based natural products in various plants

| P450 | Plant species | Function | Bioactive product | References |
|---|---|---|---|---|
| CYP71AV1 | *Artemisia annua* | Amorpha-4,11-diene C-12 oxidase | Artemisinin | Paddon et al. (2013) |
| CYP71BA1 | *Zingiber zerumbet* | α-Humulene 10-hydroxylase | Zerumbone | Yu et al. (2011) |
| CYP71AV2 | *Tanacetum cinerariifolium* | Germacrene A oxidase | Costic acid isomers | Ramirez et al. (2013) |
| CYP720B1 | *Pinus taeda* | Abietadienol/abietadienal oxidase | Oxygenated abietadienes and levopimaradiene type compounds | Ro et al. (2005) |
| CYP720B4 | *Picea sitchensis* | Multifunctional enzyme | Abietadiene and levopimaradiene type compounds | Hamberger et al. (2011) |
| CYP76AH1 | *Salvia miltiorrhiza* | Miltiradiene oxidase | Ferruginol | Guo et al. (2013) |
| CYP71D12 | *Catharanthus roseus* | Tabersonine 16-hydroxylase | Vindoline | Besseau et al. (2013) |
| CYP71D20 | *Nicotiana tobacum* | 5-Epiaristolochene1,3dihydroxylase | Capsidiol | Nguyen et al. (2012) |
| CYP93E1 | *Glycine max* | β-Amyrin 24-hydroxylase | Olean-12-ene-3β-24-diol Soyasapogenol B | Shibuya et al. (2006) |
| CYP701B1 | *Physcomitrella patens* | Ent-kaurene oxidase, chloroplastic | Ent-kaurenoic acid oxygenated | Miyazaki et al. (2011) |
| CYP716A12 | *Medicago truncatula* | β-Balsams alcohol-11-hydroxylase | Oleanolic acid, ursolic acid, betulinic acid | Carelli et al. (2011) |
| CYP725A4 | *Taxus cuspidata* | Taxadiene 5-α-hydroxylase | Taxadiene-5α-ol | Ajikumar et al. (2010) |
| CYP71D55 | *Hyoscyamus muticus* | Premnaspirodiene oxygenase | Solavetivone | Takahashi et al. (2007) |
| CYP72A1 | *C. roseus* | Secologanin synthase | Secologanin | Irmler et al. (2008) |
| CYP88D6 | *Glycyrrhiza uralensis* | β-Amyrin 11-oxidae | 11-Oxo-β-amyrin | Seki et al. (2008) |
| CYP72A154 | *Glycyrrhiza* spp. | 30-Hydroxy-11-oxo-β-amyrin | Glycyrrhetinic acid | Seki et al. (2011) |
| CYP716A47 | *Panax ginseng* | Protopanaxadiol synthase | Protopanaxadiol | Han et al. (2011) |
| CYP716A53v2 | *Panax ginseng* | Protopanaxadiol 6-hydroxylase | Protopanaxatriol | Han et al. (2012) |
| CYP716A52v2 | *Panax ginseng* | β-Amyrin 28-oxidase | Oleanane-type ginsenosides | Han et al. (2013) |

### *C. roseus*

*C. roseus* is the main source for secondary metabolites, specifically the terpenoid alkaloids, which have very important clinical use. There are about 130 terpenoid indole alkaloids (TIAs) that have been separated and recognized. Central to the TIA biosynthesis, secologanin is derived directly from the amino acid tryptophan and through several steps from 10-hydroxy-geraniol, respectively (Fig. 1). The generation of 10-hydroxy-geranoil in the iridoid pathway of *C. roseus* starts by the catalytic dehydrogenation of geraniol-10-dehydrogenase which is converted to 10-oxogeranial by redox of 10-hydroxy-geraniol, and secologanin synthase (SLS) produces secologanin at the final step (Salim et al. 2013).

TIA biosynthesis pathway is P450-dependent. The geraniol-10-hydroxylase (G10H) was composed of a P450 and two P450 reductase sub-units (Meijer et al. 1993) and belongs to the CYP76B sub-family designated as CYP76B6 (reviewed by Zhao et al. 2014). G10H from *C. roseus* belongs to the CYP72A sub-family (Vetter et al. 1992). Cloning and functional characterization on CYP72A1 from *C. roseus* revealed that it has secologanin synthase (SLS) activity (Irmler et al. 2000).

### *A. annua*

*A. annua* is an important medicinal plant from which "artemisinin" is harvested for use as anti-malarial drug. The sesquiterpene endoperoxide lactone is widely used in artemisinin-based combined therapy (ACT) to cure chloroquine-resistant malaria. Artemisinin was first isolated and identified in 1970s from *A. annua* (Paul and Mishra 2014). Artemisinin biosynthesis occurs through the sesquiterpene branch of the isoprenoid biosynthesis pathway (Wang et al. 2014). The rate-limiting step is the conversion of farnesyl diphosphate (FDP) by amorpha-4,11-dienesynthase (ADS) to amorpha-4,11-diene (Mercke et al. 2000; Bouwmeester et al. 1999), after which it is hydroxylated to yield artemisinic acid. The few last important steps are catalyzed by P450 hydroxylase (CYP71AV1) (Teoh et al. 2006; Ro et al. 2006). The artemisinic acid is presumed to be the direct precursor of artemisinin, but later studies revealed that the precursor is dihydroartemisinic acid (Brown and Sy 2004; 2006).

Transformation experiments performed on *A. annua* leaves and glandular secretory cells revealed that artemisinic alcohol can be converted into artemisinic aldehyde and dihydroartemisinic aldehyde, then subsequently into dihydroartemisinic acid (Bertea et al. 2005). They also identified that the presence of P450 hydroxylation enzyme in *A. annua* leads to the conversion of amorpha-4,11-diene into artemisinin. The amorpha-4,11-diene oxidase gene (*CYP71AV1*) which encoded a multi-functional enzyme was cloned from *A. annua* glandular hairs in 2006 (Ro et al. 2006) that catalyzes amorpha-4,11-diene to continuously produce the artemisinic alcohol, artemisinic aldehyde, and artemisinic acid (Fig. 2). The cloned *CYP71AV1* and cytochrome P450 reductase (*CPR*) gene, when co-expressed in yeast and analyzed by GC-MS, revealed increased production of artemisinic acid (Ro et al. 2006). Currently, a number of *CYP71AV1* genes have been cloned from different strains of *A. annua* (Kong et al. 2011; Ren et al. 2009).

### *T. brevifolia*

Taxol from *T. brevifolia* is a highly functional diterpenoid, effective as anti-cancer treatment of various carcinomas, melanomas, and sarcomas (Goldspiel 1997). Taxol was first isolated from the bark of the pacific yew tree, and in 1971, its oxygenated diterpenoid structure was determined (Heinig and Jennewein 2009). However, due to the limited resources of *Taxus* species, direct production of taxol from the yew trees remains a challenge and prompted the development of alternative means of production (Kingston 1991; Cragg et al. 1993).

Biosynthesis of taxol has been divided into upstream and downstream pathways (Ajikumar et al. 2010). The upstream DXP pathway is responsible for the supply of isopentenyl diphosphate (IPP) precursor, and the downstream geranylgeranyl pyrophosphate synthase (GGPPS) and taxadiene synthase (TS) are responsible for the taxol synthesis. Initial cyclization of geranylgeranyl pyrophosphate (GGPP) (universal precursor of diterpenoids) forms taxadiene, which successively undergoes a series of oxidation and hydroxylation reactions (Walker and Croteau 2001). Oxidation reactions that are mediated by P450 enzymes include seven hydroxylation and two epoxidation reactions (Croteau et al.

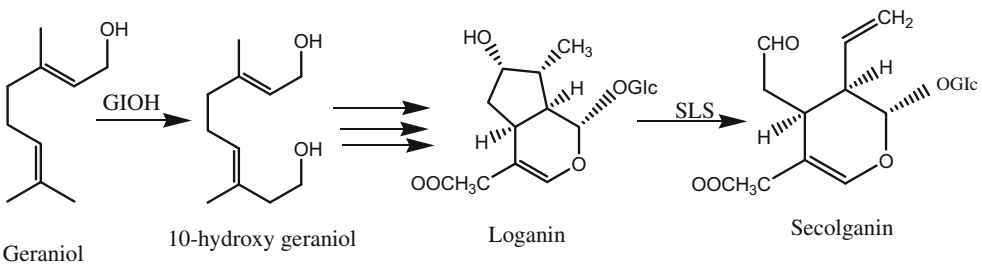


**Fig. 1** Route for TIA biosynthesis/G10H and SLS involved in TIA biosynthesis

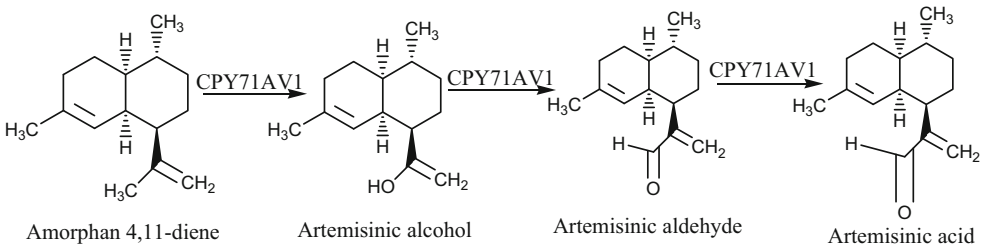


**Fig. 2** The multi-functional CYP71AV1 is involved in the biosynthesis of artemisinic acid

2006). First hydroxylation reaction occurs at the C5 position, and the addition of a hydroxyl group to C5 by taxadien-5 alpha hydroxylase also catalyzes the double bond of C4(5) to the C4(20) position (Fig. 3). The P450 taxane-10β-hydroxylase enzyme catalyzes the second hydroxylation reaction of taxadien-5α-acetoxy C10 to taxadien-5α-acteoxy-10β-ol. The third one occurs at C13 of taxadien-5α-ol to form taxa-4(20),11(12)-dien-5α,13α-diol and, consequently, taxa-4(20),11(12)-dien-5α,9α,10α,13α-tetraol. At C13 of taxadien-5α-acetoxy, hydroxylation reactions cannot occur.

Oxidation reactions of taxol to form 2α-7β-hydroxytaxusin and 7β-hydroxytaxusin and 2α, 7β-dihydroxytaxusin are catalyzed by the taxoid-2α-hydroxylase and taxoid-7β-hydroxylase (Fig. 4). In 2003, Jennewein et al. successfully cloned a cytochrome taxadiene-5α-hydroxylase (THY5a) from the yew cDNA library and catalyze the production of taxa-4(20),11(12)-dien-5α-ol from taxa-4(5),11(12)-diene and taxa-4(20),11(12)-diene when the protein was expressed in *S. cerevisiae*. Later, GGPPS, TS, cytochrome P450 taxadiene-5α (THYY5a), 10β (THY10b), 13α-hydroxylase (THY13a), taxadien-5α-ol-O-acteyle transferase (TAT), and P450 taxoid-10β-hydroxylase (THY10b) were all cloned and concurrently expressed in *S. cerevisiae* (Dejong et al. 2006).

### *Salvia miltiorrhiza*

The main lipophilic bioactive components of *Salvia miltiorrhiza* are tashinones, which are abietane-type norditerpenoid naphthoquinones (Lijuan et al. 2007). The increased demand for the tashinones is mainly due to its medical and economic importance in treating ischemic heart, tumors, diabetes, and many other infectious diseases. Tashinone is mostly synthesized in the plastid through the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway and rarely in cytosol or peroxisomes via the MVA pathway (Ma et al. 2012; Reumann et al. 2007). It follows the

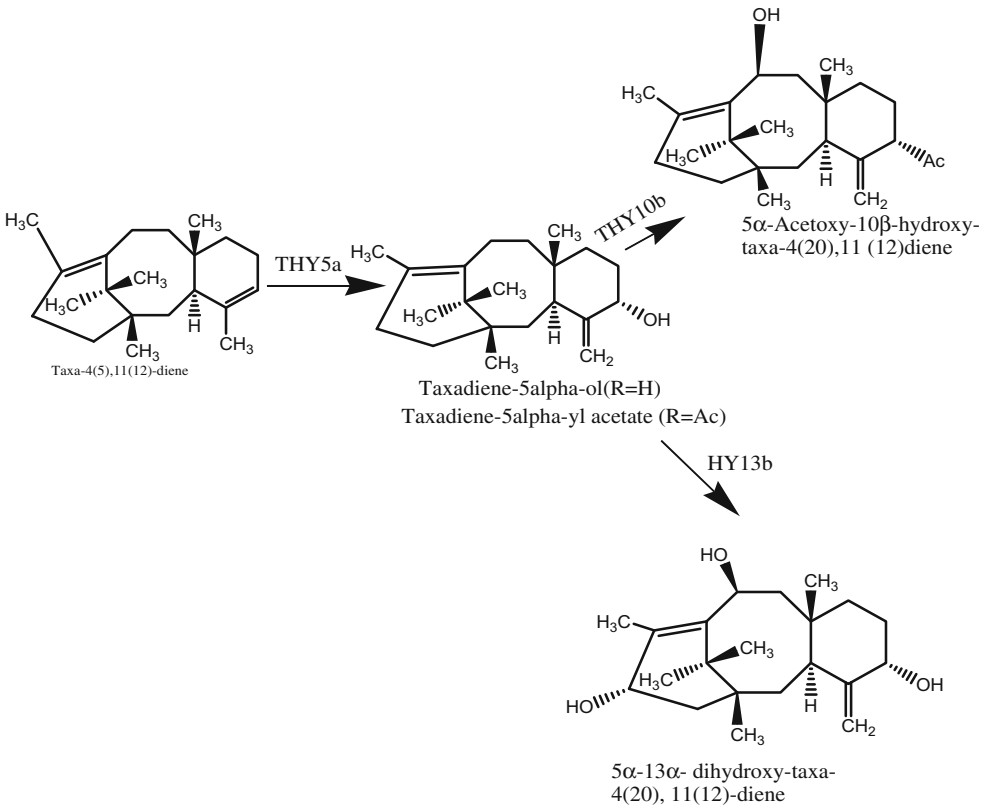


**Fig. 3** Cytochrome P450 taxadiene-5α-hydroxylase (THY5a), cytochrome P450 taxoid-10β hydroxylase (THY10b), and cytochrome P450 taxoid-13α-hydroxylase (THY13a) are involved in taxol biosynthesis

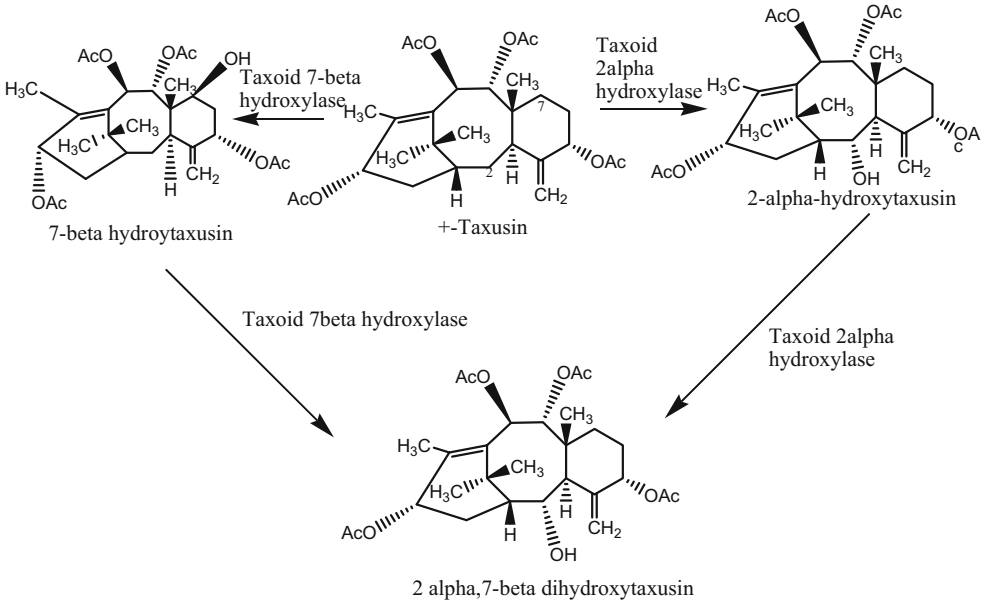


**Fig. 4** Taxoid-7β-hydroxylase and taxoid-2α-hydroxylase involved in taxol biosynthesis

regular path of transforming IPP into geranyl diphosphate (GPP), farnesyl diphosphate (FPP), and geranylgeranyl diphosphate (GGPP) (Chen et al. 2011), after which, through stepwise ionization and cycloisomerization, GGPP is converted into bicyclic labdane-type diterpenoids (Peters 2010). Various types of transferases and oxidoreductases such as copalyl diphosphate synthase (CPS), kaurene synthase-like (KSL), and cytochrome P450-dependent monooxygenases take part in a later stage (Zerbe et al. 2013; Chen et al. 2014). *SmCPS* and *SmKSL* are two diterpene synthases that catalyze the formation of miltiradiene from GGPP (Gao et al. 2009). The step where miltiradiene produces ferruginal is catalyzed by a CYP gene, CYP76AH1, in both in vitro and in vivo experiments (Fig. 5) (Guo et al. 2013). It has been shown that bioactivity of the ferruginol is similar to tashinones (Tada et al. 2000).

## Conclusion and future perspectives

During the last few years, the discovery of P450 genes has extremely accelerated; however, the functions of most P450s are still far to understand. Plant P450s are considered as the refined chemical factories against pathogens having the background of P450s tangled in the synthesis of hormones and structural building blocks. Many P450s revealed by genome sequencing projects for target drug-producing gene clusters and this evidence will provide details in the drug discovery process and give basic genetic material for plant adaptation. They have potential for the synthesis of drugs, and their manipulation leads to the generation of new drugs. Functional characterization of an accumulative number of P450 genes offers the potential to raise the fabrication of existing secondary metabolites and to obtain natural products with altered substitute patterns and composed of different building blocks.

Terpenoids are recognized as the most important class of secondary metabolites having potential applications and broad scope for their development that has made them focus of research. Cloned P450 genes have been found to play key role in their synthesis, but, in some cases, their functions remained to be explored. Manipulation by the use of genetic engineering is essential to obtain huge quantities of useful terpene compounds, similar kind of techniques should be employed to study terpenoids, and future studies should be focused on increasing their market value.

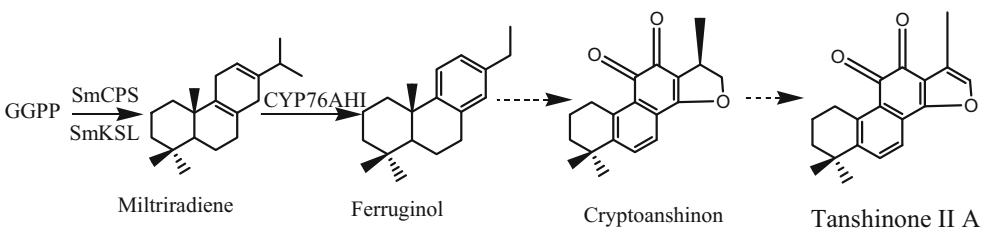


**Fig. 5** Part of putative tashinone biosynthesis pathway

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**Authors' contributions** Conceived and designed the review: SR and RM. Analyzed the data: SR. Wrote the paper: SR and RM.

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