PLANT CYTOCHROMES P450: AN OVERVIEW

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SUMMARY

Cytochromes P450 from higher plants share many general characteristics with those from animals and microorganisms. There are now 20 known P450 gene families in plants, with the number rapidly increasing. Many of these enzymes catalyze reactions in the secondary metabolic pathways of higher plants. The sheer number of plant species and the variety of these many pathways together result in the diverse enzyme chemistry available from P450s in the plant kingdom. Highlights of recent findings and of the contents of this journal issue are summarized.

DISCOVERY

Spectrophotometric evidence for cytochrome P450 in plants can be traced back to 1967 when Moore (cited in /1/) observed a CO-binding pigment with maxima at 450 and 420 nm in pea microsomes. The presence of this pigment was unequivocally linked to cinnamic acid 4-hydroxylase activity in 1974 /2,3/. As early as 1969, the presence of P450 could be inferred from the characteristics of microsomal oxidation of the terpene kaurene /4/ and of the herbicide monuron /5/. Since 1985 /6/ several plant cytochromes P450 have been purified to homogeneity. Nonetheless purification of these enzymes from plant tissues remains difficult, and other approaches have recently proven more productive. The availability of molecular biological techniques has considerably accelerated the pace of discovery. This is partially due to the economic significance of P450 oxygenases involved in the biosynthesis of commercially valuable plant metabolites (flavors, pigments, fatty acid derivatives, lignins, alkaloids, pharmaceutical precursors) and those involved in controlling resistance to both

chemical and biological stresses (herbicide tolerance and pathogen resistance, for example). At present, P450 mediated oxidation of nearly 50 endogenous physiological substrates and nearly as many xenobiotic substrates has been demonstrated. There are currently over 60 complete sequences distributed among 20 distinct cytochrome P450 gene families.

Several aspects of plant P450 have already been reviewed /1,7-11/. In this introduction we point at the main similarities and differences between plant P450 and the mammalian enzymes most readers of this journal are familiar with, and indicate those areas of plant P450 research that might be of interest to toxicologists, pharmacologists and nutritionists.

ORGANIZATION

With the notable exception of the allene oxide synthase, CYP74, most plant P450 enzymes resemble their counterparts in mammalian liver microsomes. They are proteins ranging from 48 to 60 kDa, and depend on NADPH:cyt P450 oxidoreductase to provide reducing equivalents for the monoxygenase reaction. One interesting difference is that while a single reductase, encoded by a single gene, delivers electrons to the different mammalian P450 forms, at least two NADPH-cytochrome P450 isoforms exist in several plants (Pompon, personal communication) /12,13/. The reason for this multiplicity of reductases, which can be up to 35% divergent at the peptide sequence level, remains unknown. NADPH/NADH synergism has been observed for a number but not all plant P450 reactions. Cytochrome b_5 may play a role in some cases since reconstitution of fatty acid hydroxylase activities is very low if b_5 is omitted (see Salaün and Helvig, this volume).

LOCALIZATION

Cytochrome P450 activity has been found in all plant tissues that have been assayed: seeds, tubers, roots, shoots, leaves, flowers. At the subcellular level, plant P450s are nearly always found in the microsomal fraction, functionally defined as a 100,000 *g* pellet, and are *assumed* to originate largely from endoplasmic reticulum. However, the plant microsomal fraction is a mixture of ER, peroxisomes,

glyoxysomes, Golgi membranes, plasma and vacuolar membranes, so the evidence associating the P450 with the endoplasmic reticulum is equivocal. Some spectrophotometrically detectable P450 has been found linked with several of these membrane fractions /14,15/, but where activity was measured after gradient fractionation, it was repeatedly associated with ER /16-18/. The avocado CYP71 has been shown to be associated with the endoplasmic reticulum by immunocytochemistry /19/; however, this technique has not been widely applied. Probing with antibodies against animal or microbial P450 has hinted at the existence of mitochondrial forms /20/. No P450-mediated activity has as yet been found in plant mitochondria and the P450 forms that have been sequenced present a typical 20 amino acid membrane-spanning domain at the N-terminus instead of the presequence found in the mitochondrial forms from animals.

The exceptions to this generalization occur with members of the CYP74 family, allene oxide synthases (AOS), first identified in flaxseed /21/. The flax enzyme displays a very unusual N-terminus resembling a choroplast targeting leader sequence. Tulip bulb contains a closely related AOS P450 /22/ that is isolated in the microsomal fraction, and not associated with plastid or mitochondrial fractions. The amino-terminus of this protein is unusual in that it does not contain a typical membrane anchor sequence (Lau and O'Keefe, unpublished). Another AOS, which has been purified from the membrane surrounding rubber particles in latex, shows a still different and also atypical peptide N-terminus /23/. Quite clearly the AOS P450s do not seem to be constrained to the expected subcellular localization that is found with other classes of plant P450s.

INDUCTION

The P450 content is generally low (5-50 pmoles/mg microsomal protein) and estimations are often unreliable because other plant pigments, such as flavonoids, xanthophils, carotenes and particularly chlorophylls, interfere strongly with P450 determination by the standard CO difference spectra. Like the microbial and animal oxygenases, plant P450 is highly inducible by a variety of physical and chemical agents (see Potter *et al.*, this volume). Light, acting through the photoreceptor phytochrome, considerably enhances P450 forms implicated in the synthesis of anthocyanins /18,24/ (Holton, this volume). Wounding induces the forms involved in the formation of

antimicrobial molecules and tissue cicatrization /17,25/. In fact, some isoforms catalyzing the final biosynthetic steps for phytoalexins (defense molecules) appear totally repressed in healthy plants and are detectable only in tissues or cells challenged by specific pathogens /26-30/. A wide range of chemicals have been found to induce P450 in a variety of plants. Some chemical inducers, such as barbiturates or peroxisomal proliferators, have similar effects and enhance the same type of P450 isoforms in plants as in microbes and animals (see Salaün and Helvig, and Werck-Reichhart, this volume). The best known agricultural use of chemical P450 induction is found in the use of safeners. Safeners are agrochemicals that protect crops against the deleterious effects of herbicides. It was recently found that these compounds act by selective induction of P450 (and GST) isoforms. The elevated levels of these enzymes result in enhanced detoxification of the herbicides in the crop plant /5,31/.

PURIFICATION AND RECONSTITUTION

Because of the low P450 concentration and the elevated protease content in most plant tissues, only a few P450s have been purified from plant sources. The first purified to homogeneity was a P450 from tulip /6/, later demonstrated to be an AOS /22/. Since then, P450s have been purified from a number of other sources. The successful purifications have been from rather specialized tissues (bulbs, tubers, mesocarp, *not* green leaves), and it has often been the case that the form purified was the overwhelmingly predominant P450 in that tissue /32,33/. In some tissues, etiolated wheat shoots for example, the predominant P450 appears to be an allene oxide synthase /22/, while it is clear that other distinct P450 enzymes are present in significant amounts. While the amount of these enzymes is significant on an activity basis, they may be nearly invisible as a percentage of the total cytochrome P450 measured spectroscopically.

The first step in purification of most plant P450s has involved solubilization of the microsomal membranes to get the P450 into a form in which further purification can be effected. A variety of detergents have proven successful in this and subsequent purification strategies are quite varied and well outside the scope of this paper. However, the initial solubilization also has the deleterious effect of removing the NADPH:P450 oxidoreductase protein from the proximity of the P450, and the loss of enzymatic activity during purification

is a result of this separation. There are several practical solutions to this problem: the reductase can be added to the protein to reconstitute activity, the protein can be assayed based on its ability to have a characteristic binding spectrum elicited by substrate, or an alternative oxygen donor such as an organic hydroperoxide can be used in place of O_2 and reductant. CYP74 bypasses these problems in that it is catalytically self-sufficient with a fatty acid hydroperoxide substrate.

SUBSTRATES AND REACTIONS

Substrates

One major difference between plant and animal P450s is the number of physiological substrates and the variety of reactions catalyzed by the plant enzymes. The substrates may be grossly categorized as fatty acids (see Salaün and Helvig, this volume), phenylpropanoids (see Werck-Reichhart, Holton, this volume), terpenes (see Lupien *et al.*, this volume), alkaloids, and others (see Halkier *et al.*, this volume). Table 1 shows the reactions identified so far, the systematic nomenclature when known, and a relevant reference number. In view of the bewildering number of plant-made molecules, the so called 'secondary metabolites', these 48 reactions probably represent only a fraction of the total number of P450 catalyzed reactions. Identifying these reactions and obtaining the corresponding cDNAs represent two major challenges. In addition to these endogenous substrates, plants have systems of xenobiotic detoxification that rely heavily on cytochromes P450. This is true particularly in the monocot species, which include the world's most essential crops of rice, wheat, and maize. In maize and wheat it is possible to identify 12 or more distinct herbicide metabolism reactions that are mediated by P450s (Barrett, this volume; Frear, this volume). This number is limited by the number of analogs the investigator is willing to analyze, and only represents commercially useful herbicides in a single species. Clearly other xenobiotic compounds, including other pesticides (insecticides, fungicides) and toxic waste materials, comprise known and potential substrates for these enzymes /34/. The total number of xenobiotics that are metabolized by plant P450s is probably uncountable, limited only by the numbers tested and the sensitivity of the analysis. The number of enzymes that are critical for these activities is uncertain, and will be discussed later.

TABLE 1

Plant P450 reactions with physiological substrates

Reactions, CYP nomenclature if available, reference	
Cinnamic acid 4-hydroxylase, CYP73, /2,3,36/	Ferulic acid 5-hydroxylase, CYP84, /46/
Coumaroylshikimate 3'-hydroxylase,/47/	Coumaroylquinate 3'-hydroxylase,/47/
Flavonoid 3'-hydroxylase, /24/	Flavonoid 3'5'-hydroxylase, CYP75, /48/
Isoflavone synthase /29/	Isoflavone 3'-hydroxylase, /39/
Flavone synthase II, /49/	Isoflavone 2'-hydroxylase, /39/
Pterocarpan 6a-hydroxylase, /26/	Licodione synthase, /50/
Marmesin synthase, /26/	Dianthramide B 4-hydroxylase, /42/
Psoralen synthase, /28/	Protopine 6-hydroxylase, /41/
Psoralen 5-monooxygenase, /28/	Protopine synthase, /51/
Dihydrochelirubine 12-hydroxylase, /52/	Dihydrosanguinarin 10-hydroxylase, /53/
Berbamunine synthase, CYP80, /54/	Cheilanthifoline synthase, /16/
Salutaridine synthase, /55/	Stylopine synthase, /16/
Geraniol 10-hydroxylase, /15,45/	Limonene 3-, 6- or 7-hydroxylase, /56/
Abscisic acid 6'-hydroxylase, /44/	Sabinene hydroxylase, /57/
Ipomeamarone 15-hydroxylase, /16/	Kaurene oxidases, /1/
Obtusifoliol 14a-demethylase, /37/	Glycyrrhetinic acid 24-hydroxylase, /58/
Lauric acid , (w-1),(w-3)-hydroxylase, /59, 60/	Digitalin 12-hydroxylase, /61/
Dodecane epoxygenase, /60/	Palmitic acid w-hydroxylase, /62/
Oleic w -, (w-1)-hydroxylase, /63, 64/	16-OH-Palmitate 10-hydroxylase, /65/
Tyrosine oxidase, CYP79, /66/	9,10-Epoxystearate w-hydroxylase, /63/
Allene oxide synthase, CYP74 , /21,22/	18-OH-Oleic acid 9,10-epoxidase, /67/

Many of these enzyme reactions are species specific, and in a given plant may be restricted to a specific tissue or organ, and temporally isolated to a certain developmental stage. Furthermore, as for several P450s involved in phytoalexin synthesis, activity may be detectable only after a suitable external stimulus has reached the competent tissue. Finally, and this is probably the most serious practical problem, the vast majority of the physiological substrates are not commercially available. Synthesis of the putative substrate, preferably in the radio-labeled form, and that of possible reaction products, will be a limiting factor in the exploration of these enzymes.

Over the past 2 years, the use of degenerate PCR techniques and low stringency screening of cDNA libraries has enabled the isolation of

a rapidly growing number of plant P450 clones. Most of them are "orphan" clones, not yet associated with a catalytic function. One way to examine the physiological function is to express the gene in a suitable host organism such as yeast and to assay for activity using a panel of putative substrates. This approach has a serious shortcoming, however. With no knowledge of the physiological substrate, it is impossible to distinguish between an inactive expressed enzyme and a poor substrate. At this time, purification of the protein, which is both tedious and problematic for reasons discussed above, has been instrumental in the cloning of almost all those P450s that have been clearly identified. However, two P450s have been recently cloned and identified using genetic techniques combined with an understanding of the physiological processes of the plant: the flavonoid 3'5'-hydroxylase (CYP75) (see Holton, this volume), and the ferulic acid 5-hydroxylase (CYP84) (Chapple, personal communication). A great number of mutants of all types (flowering, rooting, growth, color, biochemical mutants) are now available, notably from *Arabidopsis thaliana*, but also from many other plant species. Together with the techniques for plant transformation and regeneration that have been developed recently, this will permit the isolation and identification of many new P450s in the near future.

Reactions

Plants catalyze the same types of reactions as described for animal systems. In general these are aliphatic and aromatic hydroxylation, epoxidation, N- and S-oxidation, C-, N- and O-dealkylation, and aromatization. Additionally there is the intra-molecular oxygen transfer catalyzed by CYP74 on its fatty acid hydroperoxide substrate to form an allene oxide /21/. Another intriguing reaction is the formation of methylenedioxy bridges that are found in numerous plant compounds, many of which are defense molecules. The methylenedioxy bridge forms the active part of piperonyl butoxide, a well known insecticide synergist. These compounds function by inhibiting the P450-mediated detoxification of pyrethroid insecticides, thereby maintaining the toxicity of the insecticidal compound. It is therefore puzzling that this motif which is so efficient in inhibiting animal P450 is formed by plant P450 enzymes. Bauer and Zenk /16/ have shown that the conversion of (S)-scoulerine to (S)-stylopine, a central intermediate in the biosynthesis of diverse benzylisoquinoline alkaloids, is catalyzed by two distinct P450s (Fig. 1).

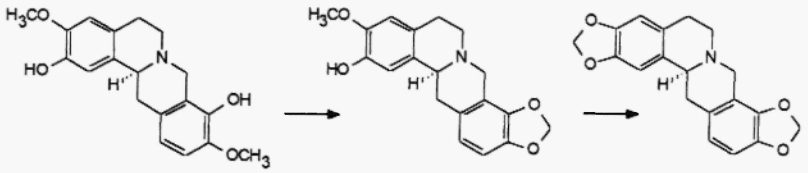
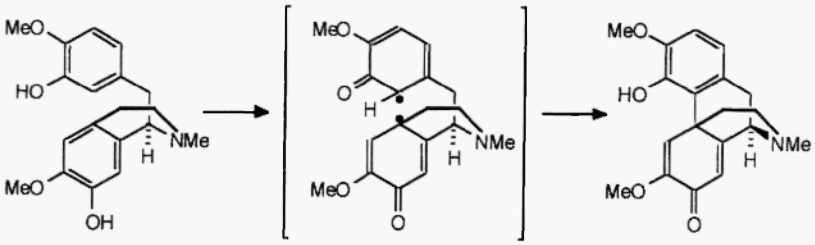


Fig. 1: The conversion of (S)-scoulerine to (S)-cheilanthifoline and to (S)-stylopine by two P450s from *Eschscholtzia californica*.

Another extraordinary reaction is the phenol oxidative coupling which forms the C-C link between carbons 12 and 13 during morphine biosynthesis /35/. The reaction is depicted in Figure 2. Although this compound can also be synthesized chemically, it is only with prohibitively low yield.



(R)-Reticuline **Salutaridine**

Fig. 2: Conversion of (R)-reticuline to salutaridine via intramolecular *para-ortho* coupling catalyzed by a P450 from *Papaver somniferum*.

THE PLANT P450 GENE FAMILIES

The first plant P450, cloned from avocado in 1990, and P450s subsequently cloned from various other plant species define 20 new gene families, as many as were discovered in animals over the last 15 years (see Durst and Nelson, this volume). Individual plant gene families may contain large numbers of members, as demonstrated by the finding that the avocado genome contains as many as 12 members of the CYP71 gene family (Christoffersen *et al.*, this volume). So far, no plant P450 is sufficiently homologous to be included as a member

of one of the microbial or animal P450 families. Although the plant P450s share less than 40% identify with other P450s, the overall characteristics appear similar to those of the microsomal enzymes from animals and yeasts. A notable exception are the CYP74 sequences. These plant hemoproteins show marked deviation from the archetypal P450 in the cys-heme coordination domain where the consensus is reduced to CxG. They also differ in the region around a conserved Thr found in the I helix of other P450s: this region is in very close proximity to the heme proximal ligand, and may be related to both the low CO affinity and the catalytic mechanism of the AOS proteins.

PLANT P450 EXPRESSION

From a physiological point of view, the plant P450s may be divided into "constitutive" and transiently expressed forms. The constitutive forms are those involved in the synthesis of major biopolymers: lignins, the cuticle and suberin. At least two P450 forms are involved in the synthesis of lignin (see Werck-Reichhart, this volume) and thus contribute substantially to biomass production. Both were cloned recently: CYP73, the cinnamate 4-hydroxylase /36/ and CYP84, the ferulate 5-hydroxylase (Chapple, personal communication). These enzymes, especially CYP84 which is more downstream in the lignin pathway, constitute important targets for the control of lignin content in fodder plants and in wood for the pulp and paper industry. The cuticle, i.e. cutin plus epicuticular waxes, covers the aerial parts (leaf, stem, flower) and constitutes the first barrier encountered by pathogens and environmental chemicals. Suberin plays a similar role for roots and also in wound healing. The elongation process of these fatty acid-derived polymers is through ester bonding between carboxylic functions and hydroxyl groups introduced at the methyl terminus by P450-linked fatty acid hydroxylases (see Salaün and Helvig, this volume). Although this has not been investigated, one may expect the presence of alkane hydroxylases to produce the long chain alcohols which are found in the epicuticular waxes and determine the hydrophobic properties of the cuticle. These fatty acid and alkane hydroxylases, which have not yet been purified or cloned, are targets for the modification of pesticide penetration or pathogen recognition and invasion. Another 'constitutive' P450 is the obtusifoliol 14-demethylase /37/ which catalyzes a key step in sterol synthesis and appears as a target for new herbicides.

Some reactions are found only in defined species. This is the case of the tyrosine N-hydroxylase involved in the synthesis of cyanogenic glucosides (see Halkier *et al.*, this volume).

Although somewhat oversimplified, we can categorize the transiently expressed forms into those expressed in defined organs according to a developmental pattern, and those expressed as a response to an external stimulus. An example of the former group are the P450s involved in the synthesis of anthocyanins, common pigments in flowers and other plant tissues. One of these, the flavonoid 3'5'-hydroxylase (CYP75) has recently been cloned /48/. The limonene hydroxylases also offer an example of closely related P450s, expressed in specialized cells, and performing the allylic hydroxylation of the same substrate but at different positions in different plants to produce different aromas (see Lupien *et al.*, this volume; Hallahan and West, this volume). Alkaloid synthesis relies on the activity of many P450 species. The examples of methylenedioxy bridge formation and of carbon-carbon coupling shown above (Figs. 1,2) are taken from exhaustive studies by Zenk and his colleagues on the biosynthesis of the benzylisoquinoline alkaloids. An impressive number of P450-catalyzed steps have been characterized during these studies, which also highlight the fact that different plant species will host common branches of a metabolic pathway (i.e. common or related P450s) and from that branch different enzymes of other pathways. For example, the C12-C13 coupling crucial for morphine synthesis was found only in microsomes from capsules of *Papaver somniferum* or from morphinane-producing papaver cell lines /35/. The preferential expression in maize inflorescences of CYP78, a P450 whose function has not yet been identified, has recently been reported /38/.

In many cases the P450s involved in the biosynthesis of phytoalexins are strongly enhanced or even become detectable only after elicitation by pathogenic challenge or exposure to elicitors. This seems to be a general phenomenon and is not related to a particular class of compounds. It concerns coumarin derivatives such as psoralens /27,28/, isoflavone derivatives /39/, pterocarpans /29/, sesquiterpenes /25/, and alkaloids such as benzophenanthridines /41/ and dianthramides /42/.

P450 also plays a role in the synthesis of plant hormones. These enzymes are under the control of both developmental programs and external factors. Three important growth regulators have P450-mediated steps in their metabolic action: a) the synthesis of gibberellins

/1/, which are diterpenoic growth hormones, involves four P450 catalyzed reactions which are under intense investigation at present; b) the catabolism of abscissic acid /43/, which is a monoterpenoic senescence hormone, and c) the synthesis of jasmonic acid /20/, a prostaglandin-like stress hormone which is catalyzed by AOS (CYP74).

SIGNIFICANCE OF PLANT P450

Soon after P450s were discovered in plants, it became apparent that they are organized very similarly to the animal microsomal system, that they are induced by xenobiotics and that they are able to metabolize these molecules. It is not surprising therefore that the 'animal' model has profoundly influenced our view of the role of these enzymes in plants. Because spectrophotometric methods failed to detect sizable amounts of P450 in adult green plants, it was proposed that its main role was detoxification of endogenous compounds in the young plant /44/. Cessation of this process would then lead to the accumulation of the secondary metabolites in the vacuoles of the mature cell. On the other hand, one was looking for xenobiotic metabolizing forms with 'broad and overlapping substrate specificity', since this is what these enzymes are well known for in mammalian drug metabolism. It appears now that most plant P450s are essentially biosynthetic enzymes and they play a vital role in the biosynthesis of a vast array of secondary metabolites. They are distributed among well over 250,000 species of flowering plants, as evolutionarily divergent as humans and dinosaurs. While much of the study of mammalian P450s has been carried out in a very small number of species, no such limitations have constrained plant researchers, and the enzymes have been studied wherever appropriate. Among this vast array of plant enzymes within these species there are many possibilities for overlapping substrate specificity between an endogenous and xenobiotic substrate. Substrate specificity will diverge between related P450s within the same family /45/, so it is also possible that the overlap between endogenous substrate and xenobiotic can change markedly between species. The broad diversity of xenobiotic metabolizing abilities to plant P450s appears to be a consequence of occasional xenobiotic overlap with an enzyme in one of the "exotic" pathways of secondary metabolite biosynthesis. This effect is magnified by the large numbers of enzymes and species under consideration.

FUTURE DEVELOPMENTS

Plant P450s are involved in major physiological pathways, including hormone and signal biosynthesis, the formation of cuticle and lignins, a wide range of secondary metabolic pathways, and detoxification of crop protection chemicals. Plant P450 enzymes represent an important potential for use in biotechnological modifications. Possible areas of future development are the use of recombinant P450 in cellular or artificial membrane reactors to catalyze the oxidation of precursors with high value such as drug precursors, the transformation of crop plants with P450 to confer resistance to defined herbicides or other systemic pesticides (see O'Keefe, this volume), engineering of plants transformed with a palette of xenobiotic metabolizing P450s as bio-remediation agents, or the use of inducible P450 promoters to monitor exposure of sentinel plants to environmental pollutants. Secondary metabolic pathways are already being closely scrutinized to find metaolic sites where the biosynthesis of high value plant products (e.g. flower color, flavor, aroma) might be modified. In the shorter term, the growing recognition of the number of P450 forms that exist in plants will allow the development of diagnostic tools to allow better characterization of the genotype of crop species, so that plant breeding can better utilize molecular genetics. An understanding of the basic biochemistry of each of these P450-mediated steps is essential to the success of these efforts.

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