CYTOCHROME P450-DEPENDENT OXIDATION OF FATTY ACIDS

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SUMMARY

Cytochrome P450-dependent monooxygenases from plants catalyse in-chain and omega hydroxylation as well as epoxidation of medium- and long-chain fatty acids. Recent research efforts have clarified that there are multiple forms of cytochrome P450 involved in these reactions, each of which possesses distinguishable substrate specificity. The biological roles of these distinct P450 forms are poorly understood. However, evidence suggests that some may play an important role in the biosynthesis of plant cuticles. We review current knowledge on the induction and inhibition of activities as well as the regio- and stereo-specificity of the distinct forms so far characterised.

KEY WORDS

plant, induction, inhibition, mechanism-based inhibitor, epoxidation, hydroxylation

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Abbreviations: CA4H, cinnamic acid 4-hydroxylase; n-DDYA, n-dodecynoic acids; 17-EODNYA, 9,10-epoxy-octadec-17-ynoic acid; FA, fatty acid; IC-LAH, in-chain lauric acid hydroxylase; (ω-1)- and ω-LAH, omega minus one- and omega-hydroxylase of lauric acid; (ω-1)- and ω-OAH, omega minus one- and omega-hydroxylase of oleic acid; n-ODNYA, 9-octadecen-n-ynoic acid; MeTMS, trimetylsylil ether methyl ester

INTRODUCTION

Plants are characterised by the presence of distinct cytochrome P450 isoforms /1,2/. Some of these appear to be tissue, organ and species specific /3-5/. Others, such as CYP73 (CA4H), are widely distributed in the plant kingdom /6/. Also, as in mammals, the involvement of multiple forms of cytochrome P450s in medium- and long-chain fatty acid (FA) oxidation is well established /1/. Interestingly, there are several similarities between mammals and plants in the catalytic mechanisms and the induction of enzyme activities by various xenobiotics. The plant P450s involved in FA oxidation have not yet been isolated because these membrane-bound enzymes are generally present in tissues at very low concentrations. During the past decade several P450s encoded by the CYP4 gene family (mainly fatty acid hydroxylases) have been purified and cDNAs have been isolated and sequenced from mammalian and insect cDNA libraries. Even though more than fifty cDNAs encoding plant P450s have been sequenced to date, none significantly matches the genes of the CYP4 family from mammals and insects.

The biological roles and the substrate specificity of cytochromes P450 involved in fatty acid and eicosanoid oxidation are poorly understood. Oxygenated FA from plants are mainly found in polar lipids such as glycerides and phospholipids /7/ and as monomers in polymeric layers. Cutins and suberins, which prevent water loss and chemical penetration and protect plants from microbial attack, are mainly constituted of hydroxylated fatty acids /8/. Some of these are potent inducers of fungal cutinase /9/ and some show anti-fungal properties /10/. These contrasting and apparently opposite effects may be due to the great diversity of defence mechanisms found in plants and also to strategies developed by the fungi to infect the plant host. On the other hand, and as reported in mammals, hydroxylated fatty acids may play a role in responding to various stresses by giving rise to inflammatory processes /11,12/ as a defence mechanism. Moreover, the presence of large amounts of ω-hydroxy derivatives of the C18 family in the plant stigma suggests that they may play a role in recognition of the stigma by pollen /13/.

Long-chain fatty acid omega and in-chain hydroxylases may play an important role in the synthesis of plant cuticles by generating hydroxy functions which appear essential to polymerisation of constitutive cutin monomers /14,15/. Cuticle monomers are often present as complex mixtures with species-specific profiles. In addition to hydroxylated FA,

expoxidated derivatives are also found as monomers of cuticles /16/ in a few plant species /17/. Moreover vicinal diol derivatives resulting from chemical and enzymatic ring-opening of epoxides have not been detected in cuticles from the C16 fatty acid family, suggesting that an epoxide function is not essential for polymerisation of the cuticle matrix. In this case it was suggested that the introduction of an internal hydroxyl group involves a direct hydroxylation mechanism catalysed by cytochrome P450-dependent fatty acid hydroxylases /18/.

FA and their derivatives are subjected to many types of oxidation reaction including hydroxylation, epoxidation, dehydration and reduction. Several forms of P450 are suspected of being involved in these reactions. For example, previous studies from our laboratory have demonstrated that at least three distinct P450 isoforms are present in microsomes from various plant species when incubated with a model substrate such as lauric acid /19-21/. An interesting feature is that these P450 systems catalyse alternatively hydroxylation and epoxidation of unsaturated laurate analogues with a regio-specificity strongly dependent on the position and stereo-specificity dependent on the configuration of the double bond in the aliphatic chain /19,22,23/. To date in-chain and ω-hydroxylating enzymes have not been found together in the same plant.

This review surveys the P450-dependent reactions involved in oxidation of fatty acids and derivatives in plants. The reactions are grouped according to type of reaction and to the position of the carbon attacked. Some examples of the induction by chemicals and inactivation by 'suicide' substrates of the P450 activities under consideration are discussed.

OMEGA-OXIDATION

Medium-chain fatty acids

A lauric acid ω-hydroxylase (ω-LAH), producing exclusively 12-hydroxylauric acid, has been described in *Pisum sativum* /21/, *Vicia sativa* /24/ and other leguminosae /25/. In addition to laurate hydroxylation, the microsomal fraction from clofibrate-treated *V. sativa* seedlings also catalysed the ω-hydroxylation of capric (C10:0) and myristic (C14:0) acids (Fig. 1). A free carboxyl group appears essential for the binding of substrates to the enzyme /21/. Induction

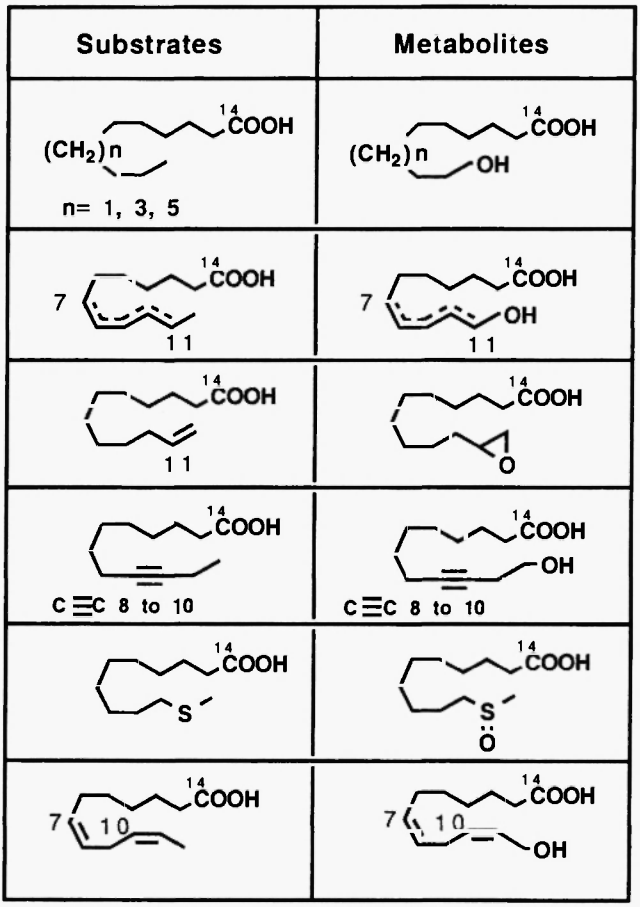

Fig. 1: Substrate specificity of the microsomal ω-LAH from *V. sativa* seedlings.

and inhibition studies strongly suggest that a single cytochrome P-450 is involved in the ω-hydroxylation of these fatty acids /23/.

To explore the catalytic capabilities of the ω-LAH, a series of [1-^{14}C] radiolabelled unsaturated lauric acid analogues (7-, 8-, 9- and 10- dodecenoic acids) was incubated with the microsomal fraction from clofibrate treated *V. sativa* seedlings. This subcellular fraction was able to catalyse the ω-oxidation of the analogues when O_2 and NADPH were present. The *cis* and *trans* forms of the four in-chain unsaturated analogues were 12-hydroxylated with similar efficiency. It

is also important to note that allylic oxidation (i.e. 12-hydroxylation of 10-dodecenoate) occurred with complete retention of the stereochemistry of the double bond and that allylic transposition was never observed. In contrast, the terminal olefin (11-dodecenoic acid) was epoxidised by the enzyme preparation. The formation of each metabolite was inhibited to the same extent when microsomes were incubated in the presence of CO, anti-P450 reductase antibodies and suicide substrates, suggesting that a single P-450 isoenzyme is able to ω-hydroxylate lauric acid, unsaturated analogues with a double bond or 1,4-pentadiene motif and to epoxidise the terminal olefin, 11-dodecenoic acid (Fig. 1) /23/. The fact that ω-LAH activity was not inhibited by oleic acid (C18:1) at a concentration 10 times higher than that of laurate suggests that it is more specific for short and medium-chain FA /26/.

Long-chain fatty acids

Early work by Soliday and Kolattukudy /27/ demonstrated the ω-hydroxylation of palmitic acid (C16:0) by a microsomal fraction from *V. faba* (Fig. 2). Inhibition of the reaction by CO suggested the

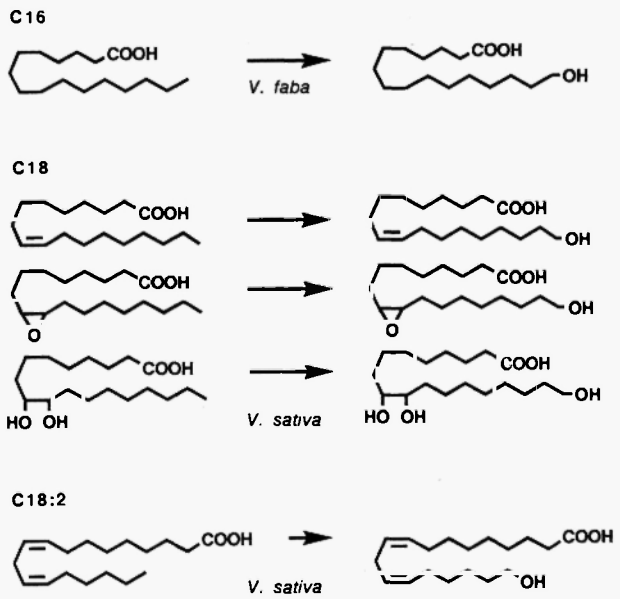

Fig. 2: Cytochrome P450-dependent ω-hydroxylation of long-chain fatty acids.

involvement of a cytochrome P-450 monooxygenase but no reversal of CO inhibition by light was obtained. More recently, microsomes from etiolated *Vicia sativa* seedlings incubated with [1-^{14}C]oleic acid (Z9-octadecenoic acid), [1-^{14}C]9,10-epoxystearic acid or [1-^{14}C]9,10-dihydroxystearic acid catalysed the NADPH-dependent formation of hydroxylated metabolites (Fig. 2). The chemical structure of these compounds was established by GC-MS analysis to be 18-hydroxyoleic acid, 18-hydroxy-9,10-epoxystearic acid and 9,10,18-trihydroxystearic acid, respectively. The reactions were inhibited by CO. Inhibition could be partially reversed by light and all three reactions were inhibited by antibodies raised against NADPH-cytochrome P450 reductase from Jerusalem artichoke /28/. The possibility that a single P450 is involved in the ω-oxidation of both oleic and linoleic acids (C18:2) is suggested by the competitive inhibition of oleic acid hydroxylation by linoleic acid and vice versa /26/.

IN-CHAIN OXIDATION

Medium-chain fatty acids

In microsomes from Jerusalem artichoke tubers (*Helianthus tuberosus*), a lauric acid in-chain hydroxylase (IC-LAH) catalyses hydroxylation of carbons 10, 9 and 8 in a 24/63/13 ratio (Fig. 3) /20/. The activity, undetectable in dormant tuber tissues, was induced by wounding and exposure to chemicals. Several other plant species, such as maize and tulip, catalyse this type of reaction, but in wheat seedlings lauric acid is mainly converted to the 11-hydroxy derivative. The lauric acid (ω-1)-hydroxylase [(ω-1)-LAH] from wheat generates a mixture of monohydroxylaurate in the proportion of 65%, 31% and 4% for 11-hydroxy, 10-hydroxy and 9-hydroxylaurate respectively (Fig. 3) /19/. Capric and myristic acids were also converted to (ω-1) and (ω-2) hydroxylated products. Additional minor metabolites hydroxylated at (ω-3) and (ω-4) were also detected when myristic acid was the substrate (unpublished data). Whatever the length of FA (C10 to C14) incubated, no ω-hydroxylated products were detected. In addition, results from our laboratory suggest that the (ω-1)-LAH from wheat catalyses the hydroxylation of the herbicide diclofop /19,29/.

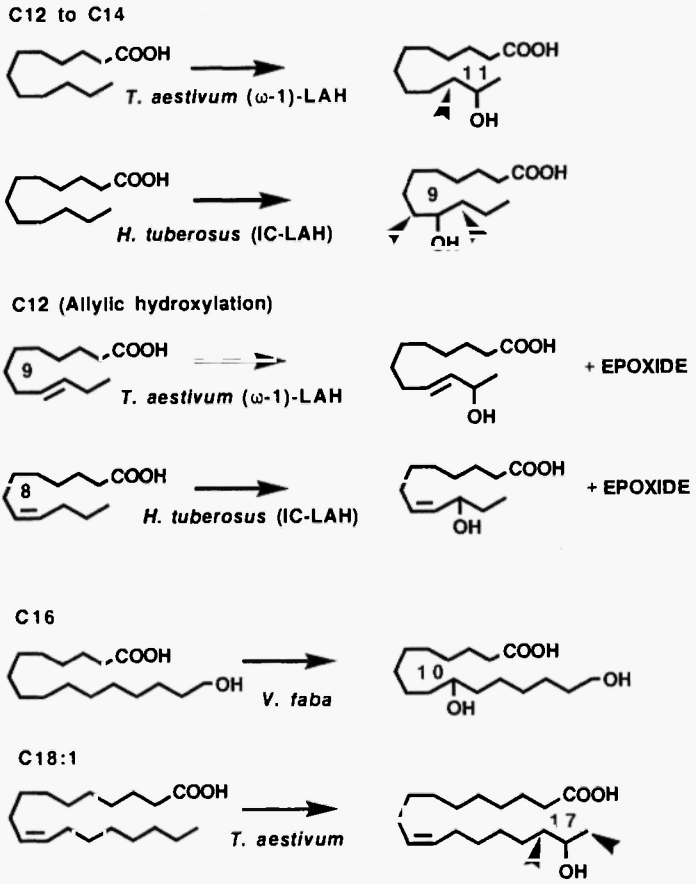

Fig. 3: Cytochrome P450-dependent in-chain hydroxylation of medium- and long-chain fatty acids.

Long-chain fatty acids

Biosynthesis of plant cuticles involves distinct P450 systems. The in-chain hydroxylation of ω-hydroxypalmitic acid /30/ by *V. faba* microsomes gives rise to 9 (or 10),16-dihydroxypalmitic acid (Fig. 3). The reactions have been attributed to a cytochrome P450 which differs from those involved in ω-hydroxylation of palmitic acid /31/ by effective reversal by light of CO inhibition.

The most abundant constituents found in the cutin of wheat caryopses are ω-hydroxylated oleic and 9,10-epoxystearic acids /32/.

Surprisingly, incubation of the microsomal fraction from etiolated wheat shoots (*Triticum aestivum* L.) with [1-^{14}C]oleic acid led to the formation of 18-, 17- and 16-hydroxyoleic acids, identified by GC-MS analysis. They were generated in a molar ratio of 1.4/4.6/4, respectively (Fig. 3). The involvement of cytochrome P450 was demonstrated by the dependence of these hydroxylations upon O_2 and NADPH, and by their light-reversible inhibition by CO. This reaction was selectively inhibited by a suitably designed mechanism-based inhibitor (see below) while lauric acid and cinnamic acid hydroxylation were not affected /33/.

SULPHUR OXIDATION

The capability of *V. sativa* microsomes to catalyse the oxidation of two sulphur-containing lauric acid analogues has been examined. Two sulphides, synthesised in radiolabelled form, [1-^{14}C]10-methylsulphinyl-decanoic acid (10S-LAU) and [1-^{14}C]8-propylsuphinyloctanoic acid (8S-LAU), were incubated with *V. sativa* microsomes under conditions promoting either P450 or peroxidase reactions. In addition to the expected peroxidative oxidation, both 8- and 10-thia fatty acids were actively converted to the sulphoxide by at least two distinct membrane bound enzymes (Fig. 4). Based on the NADPH requirement, reversal of CO inhibition and inactivation of the NADPH-dependent reactions

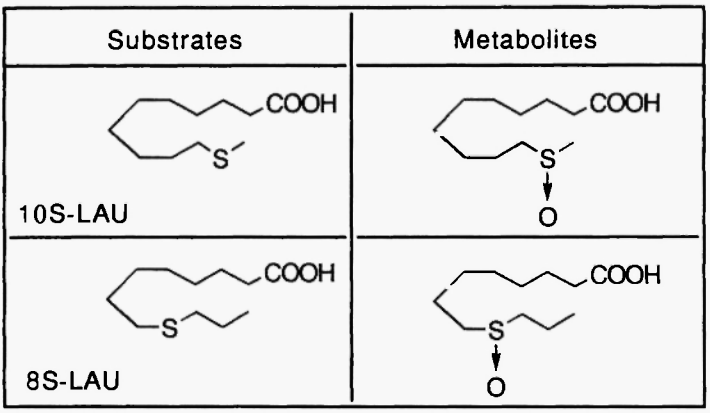

Fig. 4: Sulphoxidation of sulphur-containing fatty acids by microsomes from *V. sativa*.

by the mechanism-based inhibitor 11-dodecynoic acid (11-DDYA) targeted to inhibit the ω-LAH (see below), it was suggested that the sulphoxidation of 10S-LAU and 8S-LAU were catalysed by the same or similar P450 forms which hydroxylate lauric acid /34/. The second membrane-bound enzyme which appears to be NADPH-independent was not fully characterised. However, the presence of β-mercapto-ethanol in the incubation medium had no effect on the sulphoxidation of either 8S-LAU or 10S-LAU, suggesting that the peroxidase present in these membranes was not involved.

EPOXIDATION OF DOUBLE BONDS

Kolattukudy and co-workers fist discovered that unsaturated fatty acids are epoxidated by a subcellular fraction from plants. A 3000 *g* particulate fraction from spinach leaves catalysed epoxidation of 18-hydroxyoleic acid to 9,10-epoxy-18-hydroxystearic acid (Fig. 5) /35/. The reaction required NADPH and O_2 but surprisingly also required ATP, Mg^{++} and CoA. This unexpected cofactor requirement for P450 reactions and the lack of reversal by light of CO inhibition prevented the authors from confirming the involvement of a cytochrome P450. The formation of 9,10-epoxy-12(Z)-octadecenoic (coronaric) acid by incubation of linoleic acid with subcellular fractions from pea (*Pisum sativum*) leaves was reported to involve a P450 enzyme (Fig. 5) /36/. Interestingly, a microsomal preparation from developing endosperm of *Eurphorbia lagascae* generated 12,13-epoxy-9(Z)-octadecenoic (vernolic) acid (Fig. 5) only when linoleic acid was initially incorporated in membrane lipids (mainly into phosphatidylcholine) /37/. The epoxidase exhibits the characteristics of a cytochrome P450 and is specific for oxidation of the Δ^{12} position. Free linoleic acid was not a substrate of the epoxidase and neither oleic acid nor linoleic acid was converted to a 9,10-epoxide. A terminal olefin, 11-dodecenoic acid, was readily converted by *V. sativa* microsomes to 11,12-epoxylaurate (Fig. 5) without measurable enzyme inactivation /23/ although this compound is a mechanism-based inhibitor of ω-LAH from rat microsomes /38/.

IC-LAH from *Helianthus tuberosus* tuber, yielding mainly 9-hydroxylaurate, was demonstrated as an active epoxidase when tested with a series of unsaturated lauric acid analogues. Depending on the double bond position (from carbons 8 to 11), the enzyme was able to

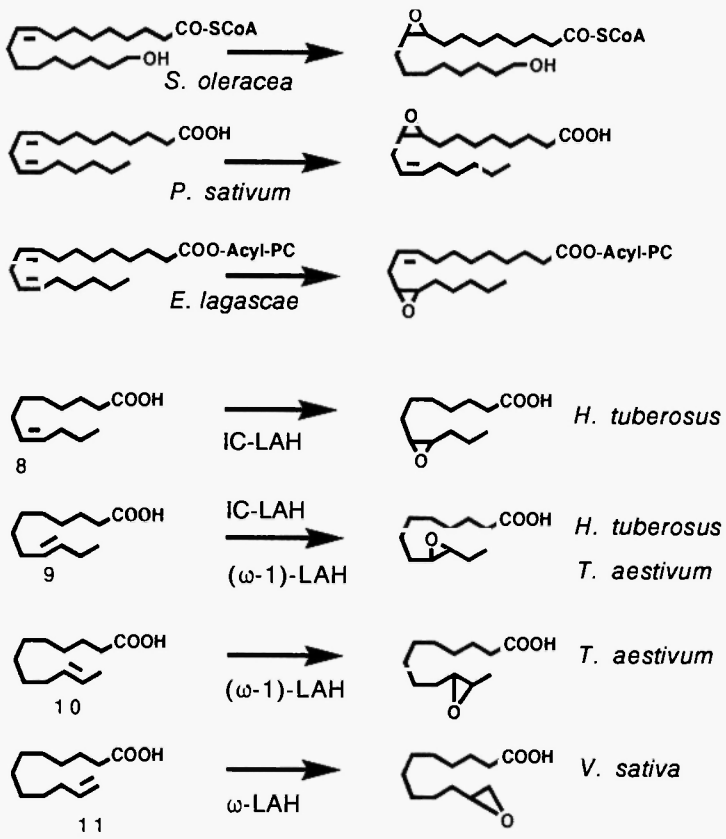

Fig. 5: Epoxidation of unsaturated fatty acids by plant P450-dependent reactions.

catalyse regio- and stereo-selective epoxidation as well as allylic hydroxylation of dodecenoic acids (Fig. 5) /23,39,40/. Similar results were obtained by incubating a microsomal (ω-1)-LAH from wheat with this series of analogues /19/.

OXIDATION OF ACETYLENIC FATTY ACIDS

Product formation from a series of labelled 8-, 9- and 10-dodecynoic acids was dependent upon NADPH and was inhibited when microsomes from *V. sativa* were incubated in a mixture of CO/O_2 (1/1 v/v) and with anti-P450-reductase antibodies, demon-

strating the involvement of P450 and P450-reductase in their metabolism. Chemical hydrogenation of metabolites and GC-MS analysis of MeTMS derivatives showed an identical mass fragmentation pattern corresponding to 12-hydroxylauric acid. It was concluded that 12-hydroxy-*n*-dodecynoic acids were originally generated by microsomes (Fig. 1). An additional uncharacterised metabolite was generated from 8- and 9-dodecynoic acids but not from 10-dodecenoic acid /34/.

In addition to saturated short and medium chain FA from C10 to C14, *V. sativa* microsomes catalyse the ω-hydroxylation of FA containing a double or triple bond with the same regio-selectivity. Evidence suggests that a single or very similar forms of P450 are involved in these reactions

DEHYDRATION OF HYDROPEROXIDE AND REDUCTION OF OXO-ACID

A new type of cytochrome P450 (CYP74A1), which converts fatty acid hydroperoxide to a metabolic precursor of jasmonic acid, was recently discovered in microsomes from flaxseed (*Linus usitanum*) /41/. The allene oxide synthase (AOS), which is unique among the known reactions catalysed by P450 enzymes, generates an unstable allene oxide (12,13-epoxy-9(Z),11(E)-octadecadienoic acid) from 13-(S)-hydroperoxylinoleic acid (Fig. 6). This new plant P450 which catalyses the dehydration of hydroperoxide with a turnover rate of 1000 per second was purified to homogeneity from an acetone powder of flaxseed and shows a molecular mass of 55 kDa in SDS-PAGE electrophoresis. Recently, rubber particle protein (RPP), a P450 from guayule (*Parthenium argentatum*) rubber particles catalysing the same reaction as AOS, was purified and cloned. The cDNA sequence of RPP shows 65% homology with that of AOS /5/. For both AOS and RPP, the reaction does not require molecular oxygen or a NADPH-cytochrome P450 reductase.

It was reported that incubation of a cell-free preparation from pea leaves with linoleic acid in the presence of NADPH leads to the formation of 12-oxo-(Z)9-dodecenoic acid resulting from the subsequent cleavage of 13-hydroperoxy-(Z)9,(E)11-octadecadienoic acid. The formation of 12-hydroxy-(Z)9-dodecenoic acid from 12-oxo-(Z)9-dodecenoic acid (Fig. 6) was attributed to a P450 reaction on the basis of CO inhibition /36/. The 12-oxo and the primary alcohol derivatives are both presumed to be plant wound hormones.

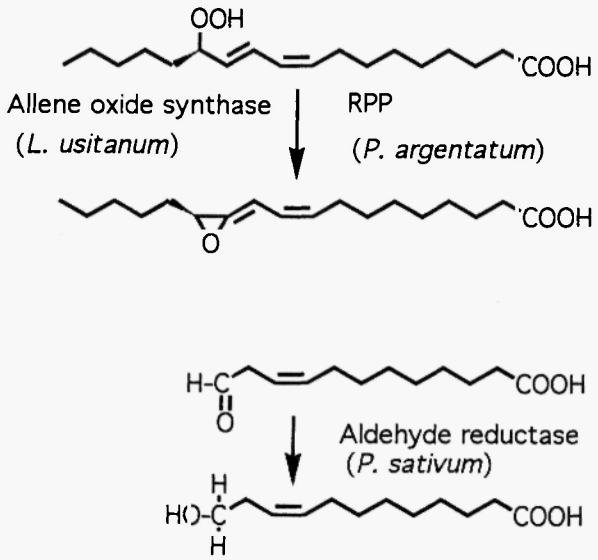

Fig. 6: Involvement of cytochrome P450 in the metabolism of fatty acid hydroperoxide and in aldehyde reduction.

INDUCTION OF FATTY ACID HYDROXYLASES

A remarkable property of living organisms is their ability to induce the activity of P450 monooxygenases in response to chemical or physical stresses. Cytochrome P450 activities from plants are induced by light /42-44/, UV-irradiation /45/, wounding /46/, ripening /47/, fungal infection /48/, elicitors /49/, endogenous compounds /50/ and numerous chemicals, including safeners /51,52/ herbicides /53-55/, drugs /19,24,40/ and pollutants /56/.

Plant P450 systems involved in ω-hydroxylation of lauric acid (ω-LAH) and oleic acid (ω-OAH) are induced by clofibrate in a dose-dependent manner /24,28/. Clofibrate is a well known hypolipidaemic drug which induces peroxisome proliferation in both mammals /57/ and plants /58/. Clofibrate and related arylphenoxy compounds, such as 2,4-dichlorophenoxyacetic acid (2,4-D), which selectively induce fatty acid ω-hydroxylase activity, have little or no effect on the activity of IC-LAH from *H. tuberosus* tubers and (ω-1)-LAH from wheat seedlings (unpublished results). *V. sativa* microsomes contain exclusively fatty acid ω-hydroxylases. In-chain hydroxyfatty acids have

never been detected in microsomes from either untreated or clofibrate- or phenobarbital-treated *Vicia* seedlings, although exposure to these xenobiotics produces a dramatic increase of ω-hydroxylase activity: circa 20 times with phenobarbital and over 30-50 times with clofibrate /24,59/.

It is noteworthy that in mammalian systems clofibrate induces the ω-hydroxylase selectively, while phenobarbital enhances (ω-1)-hydroxylation of lauric acid. The microsomal (ω-1)-LAH activity of etiolated wheat shoot was stimulated by treatment with naphthalic anhydride (NA) or phenobarbital (PB). Coating the seeds with the safener NA resulted in a 4.5-fold increase of (ω-1)-LAH activity and a 1.5-fold increase in P-450 content, while the activity of cinnamate hydroxylase (CA4H), a P450 involved in lignin synthesis, was reduced. The herbicide metabolising activity of diclofop arylhydroxylase (DIAH) was stimulated 4-fold. A much higher stimulation of the (ω-1)-LAH and DIAH was observed when the seedlings were aged on a 5 mM PB solution. Coating the seeds with NA and subsequently aging on PB resulted in a synergistic stimulation of (ω-1)-LAH and DIAH (20 times) while CA4H activity was strongly depressed. Cytochrome P450 content was increased to about 0.5 nmole.mg^{-1}, one of the highest levels so far recorded in plants. The relative amounts of 11-, 10- and 9-hydroxylaurates formed remained unchanged under all conditions /19/. Similarly, the (ω-1)-oleic acid hydroxylase activity was induced in treated seedlings to the same extend as (ω-1)-LAH, although these P450-dependent reactions were supported by distinct isoforms /33/.

A wide range of chemicals has been found to induce the IC-LAH activity of tubers and bulbs from Jerusalem artichoke, tulip and maize. Activity was induced above the untreated level by wounding slices of Jerusalem artichoke tubers in the presence of 25 mM $MnCl_2$ and 20 mM aminopyrine, but was even more enhanced when tissues were exposed to 8 mM phenobarbital /59,60/.

The mechanism of P450 induction in plant systems remains unknown but most of the P450 inducers active in mammals are also effective in induction of plant P450. It was recently demonstrated that induction of P450-dependent fatty acid hydroxylases from rodent liver by hypolipidaemic drugs, such as clofibrate, and certain physiological conditions involves transcriptional activation of the genes which was mediated by receptors (peroxisome proliferator-activated receptors). It was suggested that the perturbation of lipid metabolism is the common factor for fatty acid hydroxylase induction by peroxisomal proliferators /61/.

IRREVERSIBLE INHIBITION OF FATTY ACID ω-OXIDATION

Mechanism-based inhibitors ('suicide' substrates) containing a terminal acetylene are potent irreversible inhibitors of both plant /62,63/ and mammalian /64-67/ fatty acid ω-hydroxylases. Pre-incubation of microsomes from clofibrate-treated *V. sativa* seedlings with 11-dodecynoic acid (11-DDYA) and NADPH resulted in a pseudo-first-order loss of lauric acid ω-hydroxylation with K_i = 150 μM and a half-life of 2.4 min. The apparent rate constant for inactivation by 11-DDYA was 4.3-4.8 $x10^{-3}$ s^{-1} /63/. Recently, we incubated microsomes from *V. sativa* with [1-^{14}C]11-DDYA (Fig. 7). A major metabolite, 1,12-decanedioic acid, was probably generated by addition of water to a putative ketene intermediate (Fig. 8). This ketene may also interact with nucleophilic residues in the active site leading to a selective chemical labelling of two proteins bands (about 50 kDa). The labelling of microsomal proteins, which correlated well with diacid formation and inactivation of ω-LAH, increased as a function of incubation time and concentration of [1-^{14}C]11-dodecynoic acid (unpublished). Based on these results, two potential inhibitors targeted to inactivate the ω-hydroxylation of oleic acid were synthesised (Fig. 7). Incubation of microsomes from *V. sativa* with terminal acetylenes, (Z)9-octadecen-17-ynoic acid (17-ODNYA) and the corresponding (Z)9,10-epoxyoctadecan-17-ynoic acid (17-EODNYA), resulted in a pseudo-first-order loss of oleic acid ω-

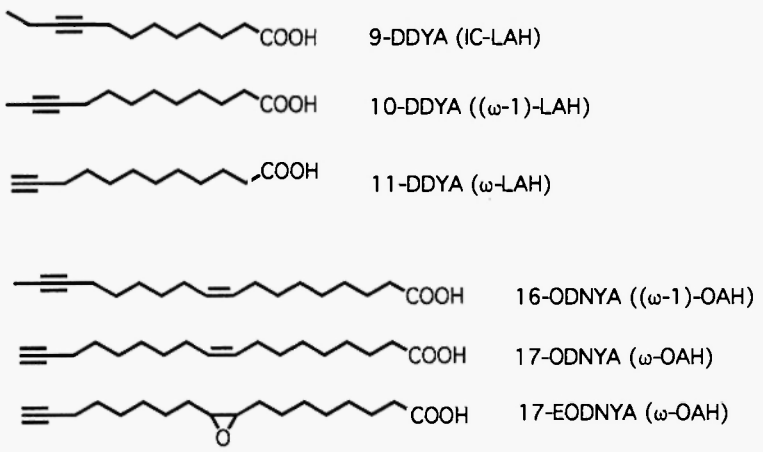

Fig. 7: Mechanism-based inhibitors of P450-dependent fatty acid hydroxylases.

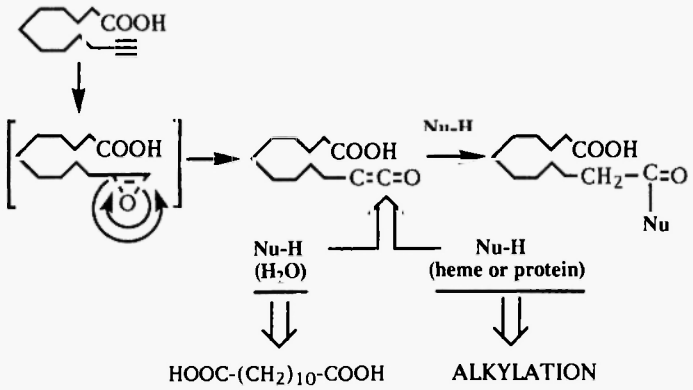

Fig. 8: Proposed mechanism of ω-LAH inactivation by 11-DDYA.

hydroxylation with apparent K_i of 60 μM and 50 μM, respectively. The calculated half-lives of enzyme activity were 6 min and 8 min for saturating concentrations of 17-ODNYA and 17-EODNYA, respectively /26/. Interestingly, these suicide substrates inhibit the ω-hydroxylation of oleic acid, epoxide and diol derivatives, and also linoleic acid to a similar extent (unpublished).

In attempts to purify and sequence plant P450s, a selective covalent binding of P450 apoproteins with labelled mechanism-based inhibitors should provide a useful means of following the labelled protein during purification steps.

INHIBITION OF FATTY ACID IN-CHAIN HYDROXYLASE

The terminal olefin 11-dodecenoic acid inactivates a P450 from wheat which catalyses mainly oxidation of the internal carbon (ω-1) of laurate /19/. As already proposed by Ortiz de Montellano and co-workers /66/, P450 inactivation by a terminal olefin proceeds via an oxidative attack on the internal carbon (ω-1) of the double bond leaving a terminal methylene radical free to alkylate the haem unit. In contrast, the plant ω-LAH which exclusively attacks the external position, catalysed the formation of the 11-12 epoxide without any measurable loss of activity /23/. Acetylenic derivatives of lauric acid are also potent inactivators of (ω-1)-LAH from wheat. Incubation of microsomes from etiolated wheat seedlings with 10-dodecynoic acid

(10-DDYA) produced a dramatic inhibition of lauric acid hydroxylation (Fig. 7). The inhibition was dependent upon time and concentration of inhibitor in a process characteristic of mechanism-based inhibitors. A half-live of 3 min and an apparent inhibition constant K_i of 14 μM were determined from pseudo-first-order kinetic studies of (ω-1)-LAH inhibition. Similar results were obtained by incubating microsomes with a terminal acetylene, 11-dodecynoic acid (11-DDYA) /34/.

In addition, the oleic acid hydroxylase ((ω-1)-OAH) from wheat, oxidising mainly the ω-1 position, was irreversibly inhibited by a substrate analogue displaying an acetylenic function at the ω-1 position. The hydroxylation of oleic acid, but not of lauric acid, was inhibited when microsomes were incubated with *cis*-9-octadecen-16-ynoic acid (16-ODNYA) (Fig. 7) /33/. These results strongly suggest that at least two different P450 enzymes are involved in the oxidation of oleic and lauric acids.

In conclusion, internal acetylene exerted an unexpected and previously unreported highly destructive effect on P450s catalysing in-chain oxidation. The mechanism of inactivation remains unknown, but the chemical rearrangement of a putative unstable acetylene epoxide, already suspected in the formation of ketene from terminal acetylene, cannot be excluded.

CONCLUSIONS

The role and significance of cytochrome P450-dependent fatty acid hydroxylases in the physiology and biochemistry of plants have not been established. Compared to those of mammals, our understanding of the catalytic mechanism and substrate specificity of plant fatty acid hydroxylases is limited (Table 1); only two P450 forms catalysing the dehydration of a fatty acid hydroperoxide have been isolated and cloned to date. Allene oxide synthase, by generating a precursor of jasmonic acid, may be a key enzyme controlling various physiological steps in plant development. In this regard, it will also be of interest to understand the physiological role of the RPP from guayule rubber particles which apparently catalyses a similar reaction. On the other hand, evidence suggests that long-chain fatty acid hydroxylases (omega and in-chain) play an important role in the biosynthesis of plant cuticles by generating terminal and internal hydroxy functions which appear

TABLE 1

Summary of reactions catalysed by plant cytochromes P450 with fatty acids

Plant species	Substrates	Products generated or carbon position oxidised (Ref.)
Vicia faba	palmitic (C16:0)	ω-OH (27)
Vicia faba	16-hydroxy C16:0	8, 9 or 10-OH (31)
Phaseolus aureus	lauric (C12:0)	ω-0H (25)
Phaseolus vulgaris	lauric (C12:0)	ω-0H (25)
Vicia sativa	C10:0-C14:0	ω-OH (21,24)
	C12:1 Δ7-10	ω-OH (23)
	C12:1 Δ11	11,12-epoxy (23)
	C12:1 triple bonds 8-10	ω-OH (34)
	C12:1 triple bond 11	1,12-dicarboxylic + inactivation (34)
	C18:1 Δ9, C18:2 Δ9,12	ω-OH (26,28)
	9,10-epoxy C18:0	ω-OH (26)
	9,10-diOH C18:0	ω-OH (26)
Pisum sativum	C10:0-C14:0	ω-OH (21)
	12-oxo-C12:1 Δ9	12-OH-C12:1 Δ9 (36)
	C18:2 Δ9,12	9,10-epoxy-C18:1 Δ12 (36)
Glycine max	C10:0-C14:0	ω-OH (25)
Triticum aestivum	C10:0-C14:0	(ω-3), (ω-2), (ω-1)-OH (mainly) (19)
	C12:1 Δ9 or 10	9,10- or 10,11-epoxy (19)
	C12:1 Δ11	11,12-epoxy + inactivation (19)
	C18:1 Δ9	(ω-2), (ω-1) (mainly), ω-OH (33)
	Diclofop	ring hydroxylation (19,29)

TABLE 1 (cont.)

Helianthus tuberosus	C10:0-C14:0	(ω-2), (ω-3) (mainly), (ω-4)-OH (20)
	C12:1 Δ8 or 9	8,9- or 9,10-epoxy-C12:0 (22,39,40)
	C12:1 Δ7 or 10	Allylic hydroxy (9-OH) (40)
Helianthus annuus	C12:0	(ω-2), (ω-3) (mainly) or (ω-4)-OH (25)
Zea mays	"	" (25)
Tulipa fosteriana	"	" (25)
Amaryllis belladonna	"	" (25)
Spinacia oleracea	18-OH-C18:1-CoA	9,10-epoxy-18-OH-C18:0-CoA (35)
Euphorbia lagascae	linoleyl-PC	12,13-epoxy-C18:1 Δ9 (37)
Parthenium argentatum	13-OOH-C18:2 Δ9,11	α and γ-ketol fatty acids (5)
Linus usitanum	13-OOH-C18:2 Δ9,11	α and γ-ketol, cyclopentenyl product (41)

essential to polymerisation of cutin monomers. Furthermore, P450s involved in epoxidation of unsaturated FA might also be involved in resistance to disease via the production of hydroxylated and epoxidated fatty acids which have been shown to inhibit the growth of pathogens. If we consider the role of oxygenated FA in fungal infections, it seems that they have contradictory effects, because it has also been reported that certain monomers from cutin (i.e. dihydroxy fatty acids and 9,10,18-trihydoxystearic acid) are potent inducers of the cutinase of several pathogenic fungi.

A remarkable property of plant P450s involved in FA hydroxylation is their high regioselectivity in epoxidation as well as in hydroxylation. Selective inhibitors containing internal or terminal acetylene could be of value as scientific tools to study the importance and the physiological role of these P450 enzymes. The possibility of following the radioactivity of enzymes inactivated by labelled suicide substrates, rather than the enzyme activity of reconstituted preparations, may also be important for P450 isolation.

From a phylogenic point of view, the apparent similarity in substrate specificity of fatty acid ω-hydroxylases from plants and

mammals in addition to the selective induction of plant hydroxylases by peroxisome proliferators and phenobarbital makes studies of the genes encoding these enzymes even more attractive.

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REFERENCES

1. Durst F, Benveniste I, Salaün J-P, Werck-Reichhart D. Function, mechanism and regulation of cytochrome P450 enzymes in plants. Biochem Soc Trans 1992; 20: 353-357.
2. Bolwell G, Bozak K, Zimmerlin A. Plant cytochrome P450. Phytochemistry 1994; 37. 1491-1506.
3. O'Keefe DP, Leto KJ. Cytochrome P-450 from the mesocarp of avocado (*Persea americana*). Plant Physiol 1989; 89: 1141-1149.
4. Hallahan DL, Lau SMC, Harder PA, Smiley DWM, Dawson GW, Pickett JA, Christoffersen RE, O'Keefe DP. Cytochrome P-450-catalysed monoterpenoid oxidation in catmint (*Nepeta racemosa*) and avocado (*Persea americana*), evidence for related enzymes with different activities. Biochim Biophys Acta 1994; 1201: 94-100.
5. Pan ZQ, Durst F, Werck-Reichhart D, Gardner HW, Camara B, Cornish K, Backhaus RA. The major protein of guayule rubber particles is a cytochrome P450 - Characterization based on cDNA cloning and spectroscopic analysis of the solubilized enzyme and its reaction products. J Biol Chem 1995; 270: 8487-8494.
6. Gabriac B, Werck-Reichhart D, Teutsch H, Durst F. Purification and immunocharacterization of a plant cytochrome P450: The cinnamic acid 4-hydroxylase. Arch Biochem Biophys 1991; 288: 302-309.
7. Sessa DJ, Gardner HW, Kleiman R, Weisleder D. Oxygenated fatty acid constituents of soybean phosphatidycholines. Lipids 1977; 12: 613-619.
8. Kolattukudy PE. Structure, biosynthesis and biodegradation of cutin and suberin. Ann Rev Plant Physiol 1981; 32: 127-138.
9. Podila GK, Dickman MB, Kolattukudy PE. Transcriptional activation of a cutinase gene in isolated fungal nuclei by plant cutin monomers. Science 1988; 242: 922-925.
10. Kato T, Yamaguchi Y, Uyehara T, Yokoyama T, Namai T, Yamanaka S. Self defensive substances in rice plant against rice blast disease. Tetrahedron Lett 1983; 24: 4715-4718.
11. Choi D, Bostock RM. Involvement of de novo protein synthesis, protein kinase, extracellular Ca^{2+}, and lipoxygenase in arachidonic acid induction of

3-hydroxy-3-methylglutaryl coenzyme A reductase genes and isoprenoid accumulation in potato (*Solanum tuberosum* L.). Plant Physiol 1994, 104: 1237-1244.

12. Harris RC, Homma T, Jacobson HR, Capdevila J. Epoxyeicosatrienoic acids activate Na^+/H^+ exchange and are mitogenic in cultured rat glomerular mesangial cells. J Cell Physiol 1990; 144: 429-437.
13. Koiwai A, Matsuzaki T. Hydroxy and normal fatty acid distribution in stigmas of *Nicotiana* and other plants. Phytochemistry 1988; 27: 2827-2830.
14. Durst F, Benveniste I, Salaün J-P, Werck D. Function and diversity of plant cytochrome P450. In: Lechner MC, ed. Cytochrome P450. Vol 6. Montrouge: John Libbey Eurotext Ltd., 1994; 23-30.
15. Kolattukudy PE. Biopolyester membranes of plants: cutin and suberin. Science 1980; 208: 990-1000.
16. Riederer M, Schönherr J. Development of plant cuticles: fine structure and cutin composition of *Clivia miniata* Reg. leaves. Planta 1988; 174: 127-138.
17. Holloway PS. Some variations in the composition of suberin from the cork layers of higher plants. Phytochemistry 1983; 22: 495-502.
18. Soliday CL, Kolattukuty PE. Midchain hydroxylation of 16-hydroxypalmitic acid by the endoplasmic reticulum fraction from germinating *Vicia faba*. Arch Biochem Biophys 1978; 188: 338-347.
19. Zimmerlin A, Salaün J-P, Durst F, Mioskowski C. Cytochrome P-450-dependent hydroxylation of lauric acid at the subterminal position and oxidation of unsaturated analogs in wheat microsomes. Plant Physiol 1992; 100: 868-873.
20. Salaün J-P, Benveniste I, Reichhart D, Durst F. A microsomal cytochrome P450-linked lauric acid monooxygenase from aged Jerusalem artichoke tuber tissues. Eur J Biochem 1978; 90: 155-159.
21. Benveniste I, Salaün J-P, Simon A, Reichhart D, Durst F. Cytochrome P-450 dependent ω-hydroxylation of lauric acid by microsomes from pea seedlings. Plant Physiol 1982; 70: 122-126.
22. Salaün J-P, Weissbart D, Durst F, Pflieger P, Mioskowski C. Epoxidation of *cis* and *trans* delta-9-unsaturated lauric acids by a cytochrome P-450 from higher plant microsomes. FEBS Lett 1989; 246: 120-126.
23. Weissbart D, Salaün J-P, Durst F, Pflieger P, Mioskowski C. Regioselectivity of a plant lauric acid omega hydroxylase. Omega hydroxylation of *cis* and *trans* unsaturated lauric acid analogs and epoxygenation of the terminal olefin by plant cytochrome P-450. Biochim Biophys Acta 1992; 1124: 135-142.
24. Salaün J-P, Simon A, Durst F. Specific induction of lauric acid ω-hydroxylase by clofibrate, diethylhexyl-phthalate and 2,4-dichlorophenoxy-acetic acid in higher plants. Lipids 1986; 21: 776-779.
25. Salaün J-P, Benveniste I, Fonne R, Gabriac B, Reichhart D, Simon A, Durst F. Hydroxylations microsomales de l'acide laurique catalysées par le cytochrome P450 chez les plantes supérieures. Physiologie Végétale 1982; 20: 613-621.
26. Pinot F, Bosch H, Alayrac C, Mioskowski C, Vendais A, Durst F, Salaün J-P. ω-hydroxylation of oleic acid in *Vicia sativa* microsomes. Inhibition by

substrates analogs and inactivation by terminal acetylenes. Plant Physiol 1993; 102: 1313-1318.
27. Soliday CL, Kolattukudy PE. Biosynthesis of cutin. ω-Hydroxylation of fatty acids by a microsomal preparation from germinating *Vicia faba*. Plant Physiol 1977; 59: 1116-1121.
28. Pinot F, Salaün J-P, Bosch H, Lesot A, Mioskowski C, Durst F. ω-Hydroxylation of Z 9-octadecenoic, Z 9,10-epoxystearic and 9,10-dihydroxystearic acids by microsomal cytochrome P450 systems from *Vicia sativa*. Biochem Biophys Res Comm 1992; 184: 183-193.
29. Zimmerlin A, Durst F. Aryl hydroxylation of the herbicide diclofop by a wheat cytochrome P-450 monooxygenase. Substrate specificity and physiological activity. Plant Physiol 1992; 100: 874-881.
30. Walton TJ, Kolattukudy PE. Enzymatic conversion of 16-hydroxypalmitic acid into 10,16-dihydroxypalmitic acid in *Vicia faba* epidermal extracts. Biochem Biophys Res Comm 1972; 46: 16-21.
31. Croteau R, Kolattukudy PE. Biosynthesis of hydroxyfatty acid polymers: Synthesis of cutin from monomer acids by cell-free preparations from the epidermis of *Vicia faba* leaves. Biochemistry 1974; 13: 3193-3202.
32. Matzke K, Riederer M. The composition of the cutin of the caryopses and leaves of *Triticum aestivum* L. Planta 1990; 182: 461-466.
33. Pinot F, Alayrac C, Mioskowski C, Durst F. Salaün J-P. New cytochrome P450-dependent reactions from wheat - Terminal and sub-terminal hydroxylation of oleic acid by microsomes from naphthalic acid anhydride and phenobarbital induced wheat seedlings. Biochem Biophys Res Comm 1994; 198: 795-803.
34. Helvig C. Conception de nouveaux inhibiteurs basés sur le mécanisme catalytique de monooxygénases à cytochrome P450. Inactivation sélective d'hydroxylases végetales d'acides gras par des analogues acétyléniques de substrats. Ph.D. Thesis. Universite Louis Pasteur, Strasbourg, 1994.
35. Croteau R. Kolattukudy PE. Biosynthesis of hydroxyfatty acid polymers: Enzymatic epoxidation of 18-hydroxyoleic acid to 18-hydroyx-*cis*-9,10-epoxystearic acid by a particulate preparation from Spinach (*Spinacea oleracea*). Arch Biochem Biophys 1975; 170: 61-72.
36. Grechkin AN, Kukhtina NV, Gafarova TE, Kuramshin RA. Oxidation of [1-^{14}C]linoleic acid in isolated microsomes from pea leaves. Plant Science 1990; 70: 175-180.
37. Bafor M, Smith MA, Jonsson L, Stobart K, Stymme S. Biosynthesis of vernoleate (*cis*-12-epoxyoctadeca-*cis*-9-enoate) in microsomal preparations from developing endosperm of *Euphorbia lagascae*. Arch Biochem Biophys 1993; 303: 145-151.
38. Kunze KL, Mangold BLK, Wheeler L, Beilan HS, Ortiz de Montellano RP. The cytochrome P-450 active site. Regiospecificity of prosthetic hemealkylation by olefines and acetylenes. J Biol Chem 1983; 258: 4202-4207.
39. Salaün J-P, Weissbart D, Helvig C, Durst F, Pflieger P, Bosch H, Mioskowski C. Stereochemistry of oxidized fatty acids generated during

catalytic oxygenation of lauric acid and unsaturated analogues by plant microsomes. FEBS Lett 1992; 303: 109-112.

40. Salaün J-P, Weissbart D, Helvig C, Durst F, Mioskowski C. Regioselective hydroxylation and epoxidation of lauric acid and unsaturated analogues by cytochrome P450 in Jerusalem artichoke microsomes. Plant Physiol Biochem 1993; 31: 285-293.
41. Song W-C, Brash AR. Purification of an allene oxide synthase and identification of the enzyme as a cytochrome P-450. Science 1991; 253: 781-784.
42. Benveniste I, Salaün J-P, Durst F. Phytochrome mediated regulation of a monooxygenase hydroxylating cinnamic acid in etiolated pea seedlings. Phytochemistry 1978; 17: 359-363.
43. Salaün J-P, Benveniste I, Reichhart D, Feyereisen R, Durst F. Product specificity and regulation of two laurate hydroxylases in higher plants. In: Coon MJ, Conney AH, Estabrook RW, Gelboin HV, Gillette JR, O'Brien PJ, eds. Microsomes, Drug Oxidation and Chemical Carcinogenesis. New York: Academic Press, 1980; 2: 721-724.
44. Toguri T, Umemoto N, Kobayashi O, Ohtani T. Activation of anthocyanin synthesis genes by white light in eggplant hypocotyl tissues, and identification of an inducible P-450 cDNA. Plant Mol Biol 1993; 23: 933-946.
45. Kato H, Kodama O, Akatsuka T. Characterization of an inducible P450 hydroxylase involved in the rice diterpene phytoalexin biosynthetic pathway. Arch Biochem Biophys 1995; 316: 707-712.
46. Benveniste I, Salaün J-P, Durst F. A wounding-induced membranous multienzyme complex hydroxylating cinnamic acid in Jerusalem artichoke tuber tissues. Phytochemistry 1977; 16: 69-73.
47. Bozak KR, O'Keefe DP, Christoffersen RE. Expression of a ripening-related avocado (*Persea americana*) cytochrome P450 in yeast. Plant Physiol 1992; 100: 1976-1981.
48. Fujita M, Oba K, Uritani I. Properties of a mixed function oxygenase catalysing ipomeamarone 15-hydroxylation in microsomes from cut-injured and *Ceratocystis fimbriata*-infected sweet potato root tissues. Plant Physiol 1982; 70: 573-578.
49. Gunia W, Hinderer W, Wittkampf U, Barz W. Elicitor induction of cytochrome P-450 monooxygenases in cell suspension cultures of chickpea (*Cicer arietinum* L.) and their involvement in pterocarpan phytoalexin biosynthesis. Z Naturforsch C 1991; 46: 58-66.
50. Hendry GAF, Jones OTG. Induction of cytochrome P-450 in intact mung beans. New Phytol 1984; 96: 153-159.
51. Moreland DE, Corbin FT, McFarland JE. Effects of safeners on the oxidation of multiple substrates by grain sorghum microsomes. Pestic Biochem Physiol 1993; 45: 43-53.
52. Thalacker FW, Swanson HR, Frear DS. Characterization, purification and reconstitution of an inducible cytochrome P450-dependent triasulfuron hydroxylase from wheat. Pestic Biochem Physiol 1994; 49: 209-223.

53. Reichhart D, Salaün J-P, Benveniste I, Durst F. Induction by manganese, ethanol, phenobarbital and herbicides of microsomal cytochrome P-450 in higher plant tissues. Arch Biochem Biophys 1979; 196: 301-303.
54. Reichhart D, Salaün J-P, Benveniste I, Durst F. Time course of induction of cytochrome P-450, NADPH-cytochrome *c* reductase, and cinnamic acid hydroxylase by phenobarbital, ethanol, herbicides and manganese in higher plant microsomes. Plant Physiol 1980; 66: 600-604.
55. Moreland DE, Corbin FT, McFarland JE. Oxidation of multiple substrates by corn shoot microsomes. Pestic Biochem Physiol 1993; 47: 206-214.
56. Narbonne JF, Garrigues P, Ribera D, Raoux C, Mathieu A, Lemaire P, Salaün J-P, Lafaurie M. Mixed-function oxygenase enzymes as tool for pollution monitoring: Field studies on the French coast of the Mediterranean Sea. Comp Biochem Physiol 1991; 100c: 37-42.
57. Gibson GG, Orton TC, Tamburini PP. Cytochrome P-450 induction by clofibrate. Biochem J 1982; 203: 161-168.
58. Palma JM, Garrido M, Rodriguez-Garcia MI, Del Rio LA. Peroxisome proliferation and oxidative stress mediated by activated oxygen species in plant peroxisomes. Arch Biochem Biophys 1991; 287. 68-74.
59. Fonne-Pfister R, Simon A, Salaün J-P, Durst F. Xenobiotic metabolism in higher plants. Involvement of microsomal cytochrome P450 in aminopyrine N-demethylation. Plant Sci 1988; 55: 9-20.
60. Salaün J-P, Benveniste I, Reichhart D, Durst F. Induction and specificity of a cytochrome P-450 dependent lauric acid in-chain hydroxylase from higher plant microsomes. Eur J Biochem 1981; 119: 651-655.
61. Gibson GG. Comparative aspects of the mammalian cytochrome P450 IV gene family. Xenobiotica 1989; 19: 1123-1148.
62. Salaün J-P, Reichhart D, Simon A, Durst F, Reich NO, Ortiz de Montellano PR. Autocatalytic inactivation of plant cytochrome P450: Selective inactivation of the lauric acid in-chain hydroxylase from *Helianthus tuberosus* L. by unsaturated substrate analogues. Arch Biochem Biophys 1984; 232: 1-7.
63. Salaün J-P, Simon A, Durst F, Reich NO, Ortiz de Montellano PR. Differential inactivation of a plant lauric acid ω- and in-chain-hydroxylases by terminally unsaturated fatty acids. Arch Biochem Biophys 1988; 260: 540-545.
64. Muerhoff AS, Williams DE, Reich NO, Cajacob CA, Ortiz de Montellano PR, Masters BSS. Prostaglandin and fatty acid ω- and (ω-1)-oxidation in rabbit lung. Acetylenic fatty acid mechanism-based inactivators as specific inhibitors. J Biol Chem 1989; 264: 749-756.
65. Ortiz de Montellano PR, Kunze KL. Self-catalyzed inactivation of hepatic cytochrome P-450 by ethynyl substrates. J Biol Chem 1980; 255. 5578-5585.
66. Ortiz de Montellano PR, Kunze KL, Beilian HS, Wheeler C. Destruction of cytochrome P450 by vinyl-fluoride, fluroxene and acetylene: Evidence for a radical intermediate in olefin oxidation. Biochemistry 1982; 21: 1331-1339.
67. Shirane N, Sui Z, Peterson JA, Ortiz de Montellano P. Cytochrome $P450_{BM-3}$ (CYP102): Regiospecificity of oxidation of ω-unsaturated fatty acids and mechanism-based inactivation. Biochemistry 1993; 32: 13732-13741.