CYTOCHROMES P450 IN PHENYLPROPANOID METABOLISM

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SUMMARY

The phenylpropanoid pathway controls the synthesis of lignin, flower pigments, signalling molecules, and a large spectrum of compounds involved in plant defence against pathogens and UV light. More than 15 P450-dependent reactions have been characterised in this pathway. Several of these reactions constitute important regulatory branching points. Indirect and direct data indicate that distinct P450s catalyse the different reactions. The coding sequences of two enzymes have recently been determined. They belong to different P450 families, CYP73 and CYP75. The second enzyme of the main pathway, cinnamate 4-hydroxylase (C4H), is the most extensively studied plant P450 and is suspected of playing an essential role in the regulation of the whole pathway.

KEY WORDS

cytochrome P450, monooxygenases, phenylpropanoids, plant defence, pigments, phytoalexins, lignin, flavonoids, pterocarpans, furanocoumarins, cinnamate 4-hydroxylase

INTRODUCTION

The phenylpropanoid pathway is a plant-specific branch of the shikimate pathway. It produces molecules derived from a C6-C3 structure, arising from phenylalanine. Phenylpropanoids account for a large proportion of the biomass. Lignins, which can reach up to 35% of dry weight in woody tissues, result from the oxidative polymerisation of cinnamyl alcohols. C6-C3 units are also incorporated into other cell wall constituents, suberins and tannins. Monomeric phenylpropanoids are widely represented in higher plants. They form several classes of compounds, including cinnamic acids, flavonoids, isoflavonoids, coumarins, stilbenes, xanthones, etc., essentially serving as flower pigments, UV and wound protectants, signalling molecules, allelochemicals and phytoalexins. Such phenolic monomers are usually found bound to sugars, quinic or shikimic acids, amines, lipids, sulphates, or terpenoids. Their synthesis is usually specific to certain organs, developmental stages, or plant species. Accumulation of phenylpropanoid derivatives can be a response to light, physical or chemical stresses or pathogen infection.

Phenylpropanoids are essentially polyhydroxylated aromatic molecules and methylated ethers of polyphenols. It is thus not surprising that many steps in their biosynthesis have been shown to rely on cytochromes P450. The products of the pathway include powerful weapons developed by plants to fight pathogenic fungi and phytophagous animals. It is of interest that such organisms have also developed P450s which allow them to detoxify these products.

THE GENERAL PHENYLPROPANOID PATHWAY

A sequence of three catalytic steps leading from phenylalanine to activated 4-coumaroyl CoA controls the flux of metabolites toward all branches of the pathway (Fig. 1). The central reaction of this sequence is catalysed by a cytochrome P450, the *trans*-cinnamate 4-hydroxylase, C4H.

C4H was one of the first plant P450s to be characterised /1-5/. A variety of assays have been developed to measure its activity, involving ^{14}C-labelled substrate and paper /2/ or thin layer chromatography /6/, ^{3}H-labelled substrate with measurement of released tritiated water /7/, spectrophotometry /8/, or HPLC /9,10/. In contrast to the soluble phenylalanine ammonia-lyase (PAL) and coumaroyl CoA-ligase, C4H

Fig. 1: The reactions of the general phenylpropanoid pathway. PAL phenylalanine ammonia-lyase; C4H cinnamate 4-hydroxylase; 4CL 4-coumarate: CoA ligase

was shown to be associated with membranes of the endoplasmic reticulum or with slightly heavier membrane fractions /5,6,11-15/. Besides membrane association, the enzyme shows many characteristics of cytochromes P450: 1) dependence on lipids /16/; 2) reaction mechanism involving NIH shift /17,18/; CO inhibition reversed by light with a maximum efficiency at 450 nm /4/; 4) dependence on molecular oxygen and NADPH /2/ (and most other studies) with a synergistic effect of NADH at low NADPH concentrations /5,6,15,19/, and low to high spin transition upon addition of substrate /3,20,21/. The overall stoichiometry of the reaction, 1:1:1 for NADPH:O_2:*trans*-cinnamate consumed, was shown to satisfy the requirements of a monooxygenase /21/. Most reports describe an optimal pH centered around 7 to 7.5 (8 in the case of sweet potato root) and Km_{app} for cinnamate ranging from 2 µM to 30 µM. The enzyme is apparently highly specific for its substrate. *trans* conformation, side chain length, side chain double bond, and carboxylate function were found to be essential for hydroxylation /4,13,22/. *cis*-Cinnamate (K_i = 340 µM) and *o*-coumarate (K_i = 34 µM) were, however, reported to inhibit competitively the parsley /22/ or pea /23/ enzymes.

Only recently has the C4H been purified to homogeneity from *Helianthus tuberosus* tuber on the basis of type I substrate interaction /24,25/, and from mung bean shoots, on the basis of reconstituted activity /26/. A purification close to homogeneity was also reported from elicited soybean cells /27,28/. Polyclonal antibodies raised against the *H. tuberosus* enzyme were shown to produce strong and selective inhibition of cinnamate 4-hydroxylation in plant microsomes and purified fractions, and allowed a single step purification of the C4H protein from several plant species /28-30/. M_r of 57,000-58,000 was determined on SDS gels for protein from all sources. Purified C4H displayed typical spectra of a low spin P450. The absorption maximum of its reduced CO-bound form was at 450 nm for the mung bean, and 448 nm for the soybean and *H. tuberosus* enzymes. The pI of the *H. tuberosus* protein analysed by bidimensional electrophoresis was > 9 /31/. Some indirect evidence suggests that C4H might be glycosylated: the detection of sugar residues during amino acid analysis /24/, the striking improvement of specificity of anti-C4H antibodies observed on periodate-treated immunoblots /28,29/, and the binding of the protein to xylose-specific antibodies and to various lectins /28,32/.

cDNA sequences coding for C4H have been isolated from *H. tuberosus* /31,33/, mung bean /34/ and alfalfa /32/. The deduced amino

acid sequences are more than 85% identical, but show less than 30% overall identity to any other P450 sequence so far reported. They were, therefore, assigned to a new P450 family named CYP73. The M_r of CYP73 polypeptides is very close to 58 kDa, their pI range between 9 and 10. Minor sequence heterogeneities among different *CYP73* clones from *H. tuberosus* or mung bean were observed. In addition, Southern blot analysis of alfalfa genomic DNA revealed several bands hybridising the non-coding regions of the alfalfa cDNA. Thus, it seems likely that allelic variants, if not multiple *CYP73* genes, coexist in the same plant.

Two *CYP73* sequences were expressed in *S. cerevisiae*. The alfalfa cDNA, expressed in a commercial system, led to the production of small but detectable amounts of protein and activity /32/. The *H. tuberosus* clone, expressed after trimming of non-coding sequences, and using an optimized vector and engineered yeast strain which overproduces P450 reductase, allowed the recovery of up to 200 pmoles (> 1%) C4H.mg^{-1} microsomal protein /35/. This yeast-expressed enzyme was found to be exceptionally active compared to plant-extracted C4H, showing a turnover of 400 min^{-1} (versus 40 min^{-1} in plant microsomes). Functional coupling of C4H with yeast reductase was shown, in this study, to be far more effective than coupling with a reductase of human origin. The first data concerning the substrate specificity of the yeast-expressed enzyme /36/ have shown that CYP73 is very selective toward endogenous substrates: the enzyme does not metabolise *p*-coumarate, benzoate, coumarin, ferulate, naringenin, 3,9-dihydroxypterocarpan or furanocoumarin precursors, nor does it transform fatty acids, geraniol, nerol, obtusifoliol or *ent*-kaurene. C4H however demethylates a natural coumarin, herniarin (7-methoxy-coumarin), and metabolises a few other small, planar molecules, including *p*-chloro-*N*-methylaniline and the herbicide chlortoluron. As expected, the metabolism of these molecules proceeds with low efficiency and significant uncoupling.

Evidence has accumulated showing that C4H is a tightly regulated enzyme. In normal plants, C4H was found expressed at high levels in young seedlings /2,4,37,38/ and in tissues undergoing active lignification /39-41/. An increase in catalytic activity is usually observed in wounded tissues /5,6,37,41-43/. This increase can be enhanced by ethylene treatment /5,43,44/. As are many enzymes of the phenylpropanoid pathway, C4H is stimulated by light and pathogen infection. Effects of white light /2,38,40,45/, blue /37/ or UV light /46/,

and phytochrome-mediated effects of red light /13/ have been reported. Increases in C4H preceded accumulation of phytoalexins and lignification in plant tissues infected with fungi /47,11,48/. Like most mammalian P450s, C4H has also been found to be responsive to treatment of plants with foreign chemicals. Enhanced activity was observed after aging *H. tuberosus* tuber tissues in the presence of Mn^{2+}, phenobarbital, ethanol, aminopyrine, herbicides and environmental contaminants /49-53/.

Regulation of C4H in relation to the accumulation of phytoalexins and phenolic cell wall precursors in plant cell suspensions and callus cultures has also been widely documented. Activity varies as a function of the age and conditions of the culture /54-56/, and irradiation with white /58,59/ or UV light /60/. In addition, cell suspension cultures treated with fungal elicitors have been largely used to model the response of C4H and of other enzymes of the phenylpropanoid pathway to fungal infection /57,61-66/.

Light-, wounding-, or infection-promoted increases in C4H activity usually occur after a lag period of 1-3 hours, and are prevented by treatment with cycloheximide, puromycin, cordycepin or actinomycin D /5,6,38,43,44,67,68/, which is indicative of *de novo* synthesis of the enzyme resulting from gene activation. Increase in C4H transcripts after wounding or treatment with xenobiotics, or fungal elicitation of cell suspension cultures, was recently confirmed using the newly isolated nucleotidic probes /32,33,53/. In wounded *H. tuberosus* tuber, accumulation of C4H transcripts proceeds without a lag phase, the amount of transcript is maximal 10 hours after slicing, and then declines rapidly /53/. Synthesis of the C4H protein proceeds after a delay of 2 to 3 hours.

C4H was the last of the general phenylpropanoid pathway enzymes to be characterised at the molecular level. The three enzymes of the general pathway are often expressed in a coordinated manner, but the mechanisms underlying such coordination still have to be determined. C4H expression and activity could be relevant to this question, since:

1) Activity of C4H measured in induced plant extracts is usually much lower than the activity of PAL. This could be indicative of a rate limiting function for C4H, but low C4H activity *in vitro* may also result from enzyme damage during plant extraction.

2) A large body of data has accumulated since the 1970s, which indicates that both substrate and product of C4H are implicated in the regulation of PAL and of other enzymes of the pathway. Reported

effects of high cinnamic acid concentrations include competitive inhibition of PAL activity /42,69-72/, prevention of PAL induction /67,68,73-77/, and removal of active enzyme by the intermediate of a proteinaceous inhibitor /76-79/. In addition, *trans*-cinnamate and *p*-coumarate have been implicated in feed-forward processes. A stimulation of the expression of PAL and chalcone synthase activities at low cinnamate was observed /76,77,80/. Exogenously supplied cinnamic acid was also reported to produce an increase in the hydroxycinnamoyl-CoA:quinate hydroxycinnamoyl transferase involved in the production of chlorogenic acid in potato /68,75/. More recently, evidence has been obtained that both cinnamate and *p*-coumarate concentrations regulate the transcription of the PAL and chalcone synthase encoding genes /80-83/. *cis*-acting elements responsive to low cinnamate and *p*-coumarate have been characterised in the CHS15 promoter /80,83/, but a careful quantification of the endogenous pools of these intermediates failed to reveal a simple relationship with gene activation in elicited alfalfa cell cultures /84/.

Tools are available which allow the depletion of the C4H pool *in vivo* and *in vitro*: 1) Inhibitors of plant haem biosynthesis have been shown to prevent an increase of C4H activity in wounded *H. tuberosus* tuber tissues /85/. Increases in other P450s and peroxidases, however, were also inhibited. At low concentrations, these inhibitors produced an increase in extractable C4H activity, possibly indicating the existence of a feed-back mechanism in C4H regulation. 2) P450 mechanism-based inhibitors provide another means to deplete the C4H pool. 1-Aminobenzotriazole and phenoxy-1-propynes were shown to be "suicide" substrates of C4H both *in vitro* and *in vivo* /36,86,87/. Preferential inactivation of C4H relative to other P450s is observed with these molecules. High concentrations, however, inactivate other P450s as well as peroxidase.

In some plants, cinnamate 4-hydroxylase activity was reported to be activated by folic acid-containing factors, and to be loosely bound to membranes /88-90/. Despite isolation of CYP73 and unambiguous demonstration of its catalytic activity, the existence of a non-P450 cinnamate hydroxylating enzyme in some plant species cannot be completely ruled out.

FLAVONOID PATHWAYS

Flavanones represent a major branch point in flavonoid biosynthesis. (2*S*)-Naringenin is a substrate for at least four plant P450s (Fig. 2).

B-Ring hydroxylation of (2*S*)-naringenin, and biogenetically derived flavonoids, largely controls flower pigmentation. Different P450s catalyse the 3′- and the 3′,5′-hydroxylation. P450 involvement in the 3′-hydroxylation was clearly demonstrated in microsomes from both parsley cell cultures /61/ and maize seedlings /91/. The enzyme is light-inducible and apparently distinct from C4H. P-450 dependence of 3′,5′-hydroxylase was only suggested by biochemical data /92/. Use of PCR and differential screening strategies, however, led to the cloning of one eggplant and two petunia genes clearly belonging to a new P450 family (CYP75) and coding for 3′,5′-hydroxylases /93-95/. The expression of the *CYP75* gene is tissue specific, and strictly dependent on UV/blue light. The biochemistry and genetics of B-ring hydroxylations are detailed elsewhere.

Conversion of the flavanone skeleton of (2*S*)-naringenin into flavone (i.e. the formation of a 2,3 double bond) is supposed to result from an oxidative attack at the 2 position, and is catalysed in some plants by a soluble 2-oxoglutarate dependent dioxygenase, flavone synthase I /96/. In osmotically stressed soybean cell suspension cultures the reaction relies on a cytochrome P450, flavone synthase II /97/. The enzyme also metabolises eriodictyol (the 3′-hydroxy derivative). It is not detected in normal or elicitor-challenged cells. Flavone synthase II also seems to be present in microsomes of flowers from Asteridae /98/. It was recently suggested that the conversion of the 5-deoxynaringenin, (2*S*)-liquiretigenin, to the retrochalcone precursor, licodione, results from 2-hydroxylation mediated by a P450, possibly by isoflavone synthase II /99/. Neither the P450 dependence of the reaction, nor the involvement of isoflavone synthase II, however, were unambiguously demonstrated.

Another type of oxidative attack on the flavanone molecule results in a rearrangement into isoflavone, with a 2,3-migration of the B-ring. This reaction controls the pathway branching point toward isoflavonoid phytoalexins, and is catalysed by a microsomal P450 (the isoflavone synthase) in microsomes from soybean /100/ and *Peuraria lobata* cell cultures /101,102/. The conversion of both (2*S*)-naringenin into genistein in soybean and liquiritigenin into daidzein in *P. lobata* was shown to proceed in two steps, with production of a 2-

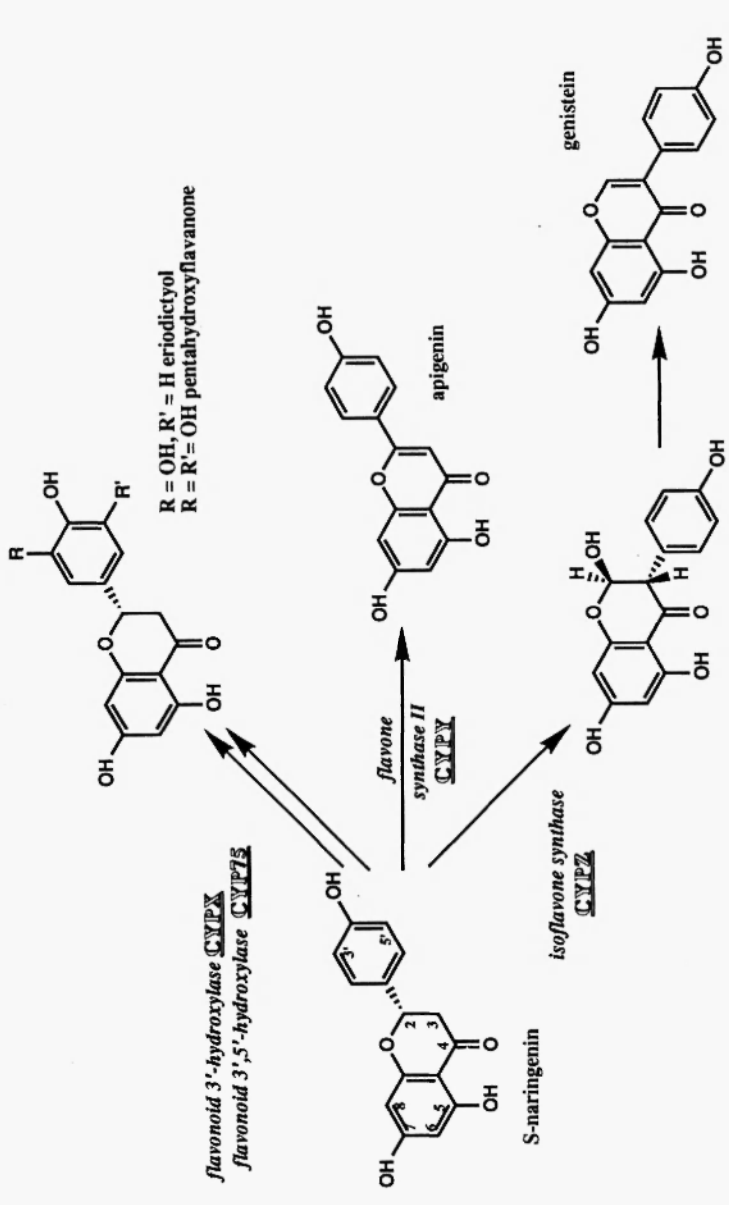

Fig. 2: Transformations of naringenin catalysed by cytochromes P450. CYP75, CYPX, CYPY, and CYPZ designate different P450 enzymes. Only genes coding for CYP75 have been isolated.

hydroxyisoflavanone rapidly dehydrated by a soluble enzyme. Experiments with $^{18}O_2$ and ^{18}O-labelled liquiritigenin suggested that the P450-catalysed formation of the 2-hydroxyisoflavanone proceeds via abstraction of hydrogen at C-3 followed by the 2,3-shift of the B-ring, and the formation of a carbon radical at C-2 which is then hydroxylated /101/. Large increases in isoflavone synthase activity in soybean cells and seedlings were observed following treatment with a fungal elicitor /103/.

Evidence of the involvement of distinct P450 isoforms in naringenin metabolism was obtained from induction experiments with soybean cell cultures. Isoflavone synthase was induced by elicitor treatment, and decreased upon osmotic shock. Conversely, flavone synthase II, absent in elicitor treated cells, was strongly induced by osmotic stress /57/. None of the treatments led to detectable B-ring hydroxylase activities in the cells. Interestingly, these different P450s selectively metabolised the (2*S*)-isomer, but only for isoflavone synthase was an inhibition of the reaction by the (2*R*)-isomer reported /100/.

After isoflavone synthase, several other P450s have been characterised in the branch leading to the synthesis of pterocarpan phytoalexins (Fig. 3).

The conversion of isoflavones to pterocarpans is initiated by a 2′-hydroxylation of the B-ring. Experiments performed with resistant and susceptible chickpea cultivars suggest that this branch point reaction controls pterocarpan accumulation and is essential for an efficient plant defence against fungal pathogens /10,66/. Involvement of P450 in 2′-hydroxylation of both formonetin and biochanin A was demonstrated in chickpea microsomes /104,105/. A P450-dependent 3′-hydroxylation of both substrates was also detected in these microsomes. This hydroxylation is required for the formation of a methylenedioxy bridge on the B ring, and the synthesis of maackiain, one of the major phytoalexins accumulated in chickpea. Only isoflavones methylated in the 4′-position are accepted as substrates by both the 2′- and 3′-hydroxylases from chickpea /104/. Differences in pH optima, sensitivity toward inhibitors and $MnCl_2$ inducibility suggest the involvement of distinct P450s in the 2′- and 3′-hydroxylations. In addition, different constitutive expression indicates that different P450 isoforms hydroxylate 3′-formonetin and biochanin A /105/. Transient induction of the isoflavone 2′-hydroxylase, together with isoflavone synthase and C4H, was observed in elicited alfalfa cell cultures which produced large amounts of the pterocarpan phytoalexin medicarpin /106/.

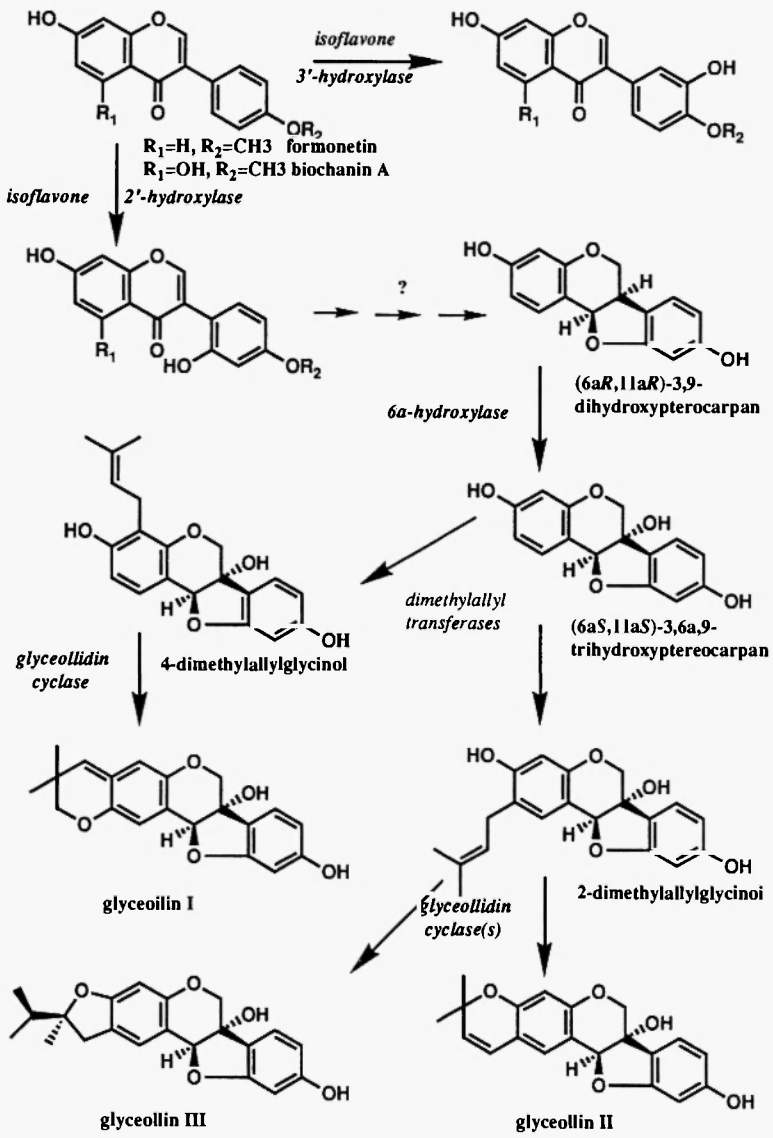

Fig. 3: P450-dependent reactions in the isoflavonoid pathway. The P450-catalysed reactions are indicated in bold italics. The substrate specificity of the 2′-hydroxylase may differ from plant to plant. In chickpea, which accumulates 4′-methoxylated isoflavonoids, 4′-methoxyisoflavones are much better substrates than the corresponding 4′-hydroxy derivatives. In other plants, however, (for example in soybean) 4′-hydroxy isoflavonoids are the major phytoalexins.

Glyceollin isomers are the major pterocarpan phytoalexins formed in soybean upon infection. Their synthesis involves a stereospecific hydroxylation of (6a*R*,11a*R*)-3,9-dihydropterocarpan into (6a*S*,11a*S*)-3,6a,9-trihydroxypterocarpan (or glycinol) catalysed by a P450 present in elicitor-treated soybean cell cultures and shoots /107/. Glycinol is subsequently prenylated in positions 2 or 4, and the 2- and 4-dimethylallylglycinols obtained are cyclised with formation of a 2,2-dimethylchromen ring (to form glyceollin I and II), or formation of a 2-isopropenyldihydrofuran ring (glyceollin III). Welle and Grisebach /108/ have demonstrated that the three cyclisation reactions are catalysed by P450 in elicitor-challenged cells. The exact number of isoforms involved, however, has not been determined. Both 3,9-dihydroxypterocarpan 6a-hydroxylase and cyclase activities are very low or undetectable in unstimulated cells. Accumulation of the pathway end-products possibly regulates the terminal enzyme(s), since a strong inhibition of the cyclase by glyceollin has been observed.

Soybean 3,9-dihydroxypterocarpan 6a-hydroxylase was one of the first plant P450s to have been purified to homogeneity /27/. The purified protein showed an M_r of 55,000 on SDS-PAGE and was enzymatically active when reconstituted with reductase and phospholipids. Cross-reactivity of the purified protein with various lectins and with anti-xylose antibodies strongly suggests glycosylation and raises the possibility of its localisation in membrane compartments involved in secretion processes /28/. C4H was clearly separated from the pterocarpan hydroxylase during purification, which provided the first direct proof of the existence of multiple P450s in higher plants. Polyclonal antibodies directed against 3,9-dihydroxypterocarpan 6a-hydroxylase have been obtained, but its coding sequence has not yet been determined.

THE FURANOCOUMARIN PATHWAY

Linear furanocoumarins are phytoalexins produced by many plants, in particular by Apiaceae, in response to pathogen attack. Three successive steps in their biosynthesis have been shown to involve P450 localised on the endoplasmic reticulum in parsley and/or *Ammi majus* elicited cell cultures (Fig. 4): 1) marmesin synthase catalysing the cyclisation of demethylsuberosin into (+)marmesin in a reaction very similar to the cyclisation of prenylated pterocarpans during the synthesis of the soybean phytoalexin glyceollin /109/; 2) psoralen

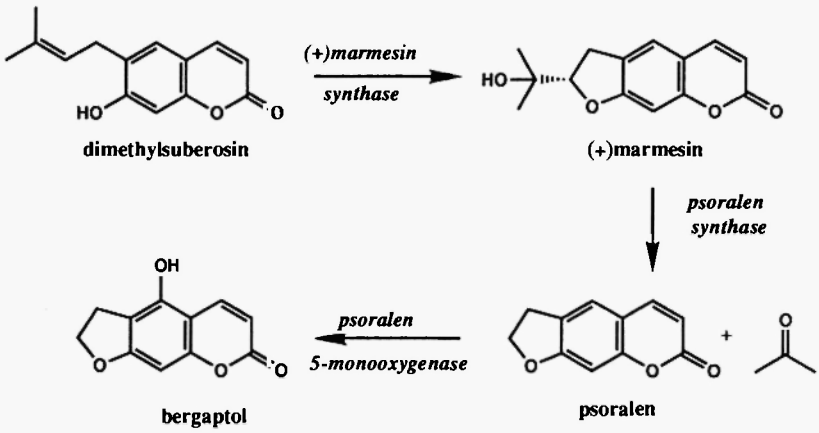

Fig. 4: P450-dependent reactions in the furanocoumarin pathway. The formation of (+)marmesin was proposed to proceed via the epoxide and the (*R*)-diol, but such intermediates have never been isolated.

synthase which specifically converts (+)marmesin into psoralen /109,110/; and 3) psoralen 5-monooxygenase leading to formation of bergapten /111/. Differences in enzyme inhibition, localisation, and in the time courses of induction after elicitor treatment, suggest the involvement of three distinct P450 isoforms in these reactions.

OTHER PHENYLPROPANOID DERIVATIVES (FIG. 5)

The synthesis of lignin monomers involves two steps of hydroxylation of the *p*-coumarate ring in positions 3 and 5.

3-Hydroxylation is usually assumed to be catalysed by soluble phenolases. A P450 with 5-*O*-(4-coumaroyl)shikimate/quinate 3-hydroxylase activity, however, has been characterised in both parsley and carrot cell suspension cultures /112,113/. The enzyme is induced by blue/UV light or fungal elicitor, and is more specific than phenolases. It was thus proposed that the 3-hydroxylation of *p*-coumarate may occur via the shikimate or quinate esters. However, a third possible pathway involving *trans*-coumaroyl CoA and a soluble ascorbate- and Zn^{++}-dependent enzyme has been described /114/. In carrot cells, the 5-*O*-(4-coumaroyl)-shikimate/quinate 3-hydroxylase also leads to the synthesis of chlorogenic acid (5-*O*-caffeoyl-D-quinic

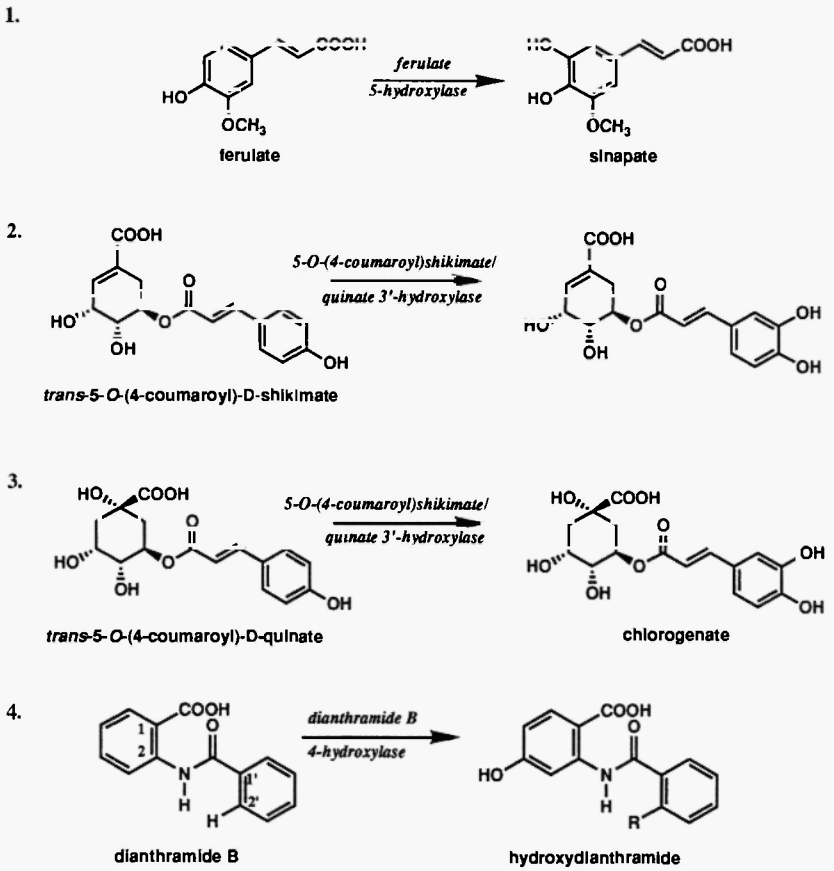

Fig. 5: P450-catalysed transformations of other phenylpropanoid derivatives. **2-3**. The *trans*-5-*O*-(4-coumaric) esters of quinate and shikimate are apparently both 3'-hydroxylated by the same P450 in carrot microsomes. 4-Coumarate or the *cis*-derivatives are not substrates. **4**. The 2'-hydroxylated dianthramide B (dianthramide S) is also 4-hydroxylated in carnation microsomes. The hydroxylated molecules are subsequently methylated to provide the final phytoalexins.

acid), accumulated in many plants and reported to be implicated in growth regulation and disease resistance /113/.

The 5-hydroxylase of ferulic acid was characterised as a P450 in microsomal fractions from poplar stems /115/. The enzyme apparently controls the monomer composition of lignins in a tissue specific

manner (5-*O*-monomers are thought to decrease the cross-linking opportunities in the lignin polymer, thus improving digestibility as well as cellulose extraction). *Arabidopsis* mutants deficient in ferulate 5-hydroxylase have recently been isolated by Chapple and coworkers /116/, providing a tool for the cloning and manipulation of its gene.

Dianthramides B and dianthalexin are phytoalexins accumulated by carnation plants or elicited cell cultures. These molecules derive from *N*-benzoylanthranilate which is 4- and/or 2′-hydroxylated. 4-Hydroxylation of the molecule was shown to involve a P450, while 2′-hydroxylation is apparently mediated by another class of enzyme /117/.

CONCLUSIONS

At least sixteen P450s have been characterised in the phenylpropanoid pathway, catalysing not only hydroxylations, but also dealkylations, cyclisations or ring migration. Most substrates and products of these enzymes are phytotoxic molecules. This raises questions about both their regulation and localisation. Only preliminary data are available concerning regulation. These data suggest expression is very strictly regulated by tissue, developmental stage and/or response to environmental signals. All enzymes were reported to be localised in microsomes and in fractions of densities similar to endoplasmic reticulum. The small density shifts observed in several experiments, scarce data on the occurrence of membrane-associated functional complexes /118/, and the apparent glycosylation of some purified proteins, however, suggest a more complex distribution. Considering the toxicity of many metabolites it would not be surprising to find some enzymes, in particular those processing the end-chain products, associated with compartments directly involved in secretion or storage processes.

Only some branches of phenylpropanoid metabolism have been explored in a small number of plants. The mechanisms involved in the biosynthesis of many products have not yet been elucidated. It is thus obvious that additional P450s will be characterised in the pathway. Moreover, important variations in the isoforms present in different plant species are expected. Phylogenetic studies on the P450s of phenylpropanoid metabolism, therefore, can be anticipated to help in clarifying plant taxonomy and evolution.

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