Phytochem Rev (2006) 5:239–270
DOI 10.1007/s11101-006-9025-1

Cytochromes P450 in phenolic metabolism

Jürgen Ehlting · Björn Hamberger · Rachel Million-Rousseau · Danièle Werck-Reichhart

Received: 30 March 2006 / Accepted: 25 August 2006 / Published online: 17 November 2006

Abstract Three independent cytochrome P450 enzyme families catalyze the three rate-limiting hydroxylation steps in the phenylpropanoid pathway leading to the biosynthesis of lignin and numerous other phenolic compounds in plants. Their characterization at the molecular and enzymatic level has revealed an unexpected complexity of phenolic metabolism as the major route involves shikimate/quinate esters and alcohol/aldehyde intermediates. Engineering expression of *CYP73*s (encoding cinnamate 4-hydroxyl ase), *CYP98*s (encoding 4-coumaroylshikimate 3′-hydroxylase) or *CYP84*s (encoding coniferaldehyde 5-hydroxylase) leads to modified lignin and seed phenolic composition. In particular CYP73s and CYP98s also play essential roles in plant growth and development, while CYP84 constitutes a check-point for the synthesis of syringyl lignin and sinapate esters. Although recent data shed new light on the main path for lignin synthesis, they also raised new questions. Mutants and engineered plants revealed the existence of (an) alternative pathway(s), which most likely involve(s) different precursors and oxygenases. On the other hand, phylogenetic analysis of plant genomes show the existence of P450 gene duplications in each family, which may have led to the acquisition of novel or additional physiological functions in planta. In addition to the main lignin pathway, P450s contribute to the biosynthesis of many bioactive phenolic derivatives, with potential applications in medicine and plant defense, including lignans, phenylethanoids, benzoic acids, xanthones or quinoid compounds. A very small proportion of these P450s have been characterized so far, and rarely at a molecular level. The possible involvement of P450s in salicylic acid is discussed.

Keywords Cytochrome P450 monooxygenases · Phenylpropanoid metabolism · Cinnamate 4-hydroxylase · C4H · Coumaroyl-shikimate 3′-hydroxylase · C3′H · Coniferaldehyde 5-hydroxylase · CA5H · F5H · Lignin · Sinapate esters · Rosmarinic acid · Salicylic acid · Benzoic acid · Podophyllotoxin · Xanthone

J. Ehlting (✉) · R. Million-Rousseau · D. Werck-Reichhart
Départment Réponses Métaboliques, Institut de Biologie Moléculaire des Plantes, CNRS-UPR2357, Université Louis Pasteur, 28 rue Goethe, F-67000 Strasbourg, France
e-mail: juergen.ehlting@ibmp-ulp.u-strasbg.fr

B. Hamberger
Department of Botany, University of British Columbia, Vancouver, BC, Canada

Introduction

Phenylpropanoid and other phenolic compounds fulfill tremendously diverse functions during plant

development and in response to environmental cues. Quantitatively the most important, lignin is a hydrophobic polymer allowing high pressure water transport and erect growth of land plants, but other phenolpropanoids play equally pivotal roles as flower pigments and fragrances ensuring reproduction, as phytoalexins and deterrents in the battle against herbivores and pathogens, as UV- and wound protectants and as signaling molecules both within the plant and in communications with other organisms (Hahlbrock and Scheel 1989; Weisshaar and Jenkins 1998; Dixon 2001; Boerjan et al. 2003). Phenylpropanoids also may have impact on human health and disease as poisons and pharmaceuticals (Graf et al. 2005; Saleem et al. 2005), and determine the quality of many products employed by humans such as wood and paper (Baucher et al. 2003). Phenolic compounds are derived from the shikimate pathway leading to the biosynthesis of aromatic amino acids. Most are derived from phenylalanine via the core phenylpropanoid pathway leading to 4-coumaroyl-CoA (Fig. 1). From there, branches dispatch precursors to the biosynthesis of the diverse phenylpropanoid classes, including monolignols, flavonoids, coumarins, stilbenes, xanthones, phenolic esters and benzoic acid derivatives.

Cytochrome P450 monooxygenases (P450s) catalyze per se slow reactions (low k_{cat}) in all these branch pathways and are frequently located at strategic positions. Given the high exothermy of the reaction catalyzed, P450s constitute ''points of no return'' in metabolic networks and thus channel flow irreversibly into the divergent branch pathways. It is therefore not surprising that more than sixteen P450s have been implicated in phenylpropanoid metabolism (Werck-Reichhart 1995).

Within this special issue, other reviews will cover the involvement of P450s in flavonoid/anthocyanin biosynthesis and thereof derived phytoalexins, their role in (furano)coumarin biosynthesis, and their potential in anchoring metabolic channels to the endoplasmatic reticulum (Ayabe and Akashi 2006; Tanaka 2006; Bourgaud et al. 2006; Ralston and Yu 2006; this issue). Also, recent reviews have covered many aspects of phenylpropanoid metabolism (Dixon, 2001; Humphreys and Chapple 2002; Anterola and Lewis 2002;. Boerjan et al. 2003; Rogers and Campbell 2004), and thus this review will focus on recent advances regarding the three hydroxylases involved in the general phenylpropanoid pathway, namely cinnamate 4-hydroxylase (C4H), coumaroyl-shikimate 3′-hydroxylase (C3′H) and coniferaldehyde 5-hydroxylase (CA5H). Further attention will be drawn to the less well characterized pathways leading to hydroxybenzoate derivatives including xanthones and ubiquinone, to hydroxyphenylethanol derivatives (salidroside/cornoside), and to lignan derivatives.

The core phenylpropanoid pathway

Phenylpropanoids are synthesized from phenylalanine via the sequential action of phenylalanine ammonia lyase (PAL) and cinnamate 4-hydroxylase (C4H) to form 4-coumarate (or *para*-coumarate), which is then activated to the coenzyme A-thioester by 4-coumarate:CoA ligase (4CL). 4-Coumaroyl-CoA is the precursor for many phenylpropanoid compounds including flavonoids, stilbenes, coumarins, and lignin (Fig. 1). The latter is constituted mainly of subunits derived from coniferyl (G-lignin subunits) and sinapyl (S-lignin) alcohols. To form these, and the corresponding aldehydes (coniferaldehyde and sinapaldehyde) and acids (ferulate and sinapate), the aromatic ring of 4-coumarate needs to be hydroxylated at the 3- and 5-positions respectively, followed by methoxylation of the hydroxy groups. It was long thought that these hydroxylations occur at the level of the free acids, but, in recent years, it became clear that the predominant pathway proceeds via 3′-hydroxylation of 4-coumaroyl-shikimate, and 5-hydroxylation of coniferaldehyde. It was also shown that methoxylations occur mainly on the level of the CoA ester and aldehyde respectively, catalyzed by caffeoyl-CoA *O*-methyltransferase (CCoA OMT) and 5-hydroxyconiferaldehyde *O*-methyltransferase (COMT). This ''rewriting the lignin roadmap'' (Humphrey and Chapple 2002) was mainly achieved by characterizing the 3′- and 5-hydroxylation steps, and it is now clear that all three hydroxylations in the core phenylpropanoid

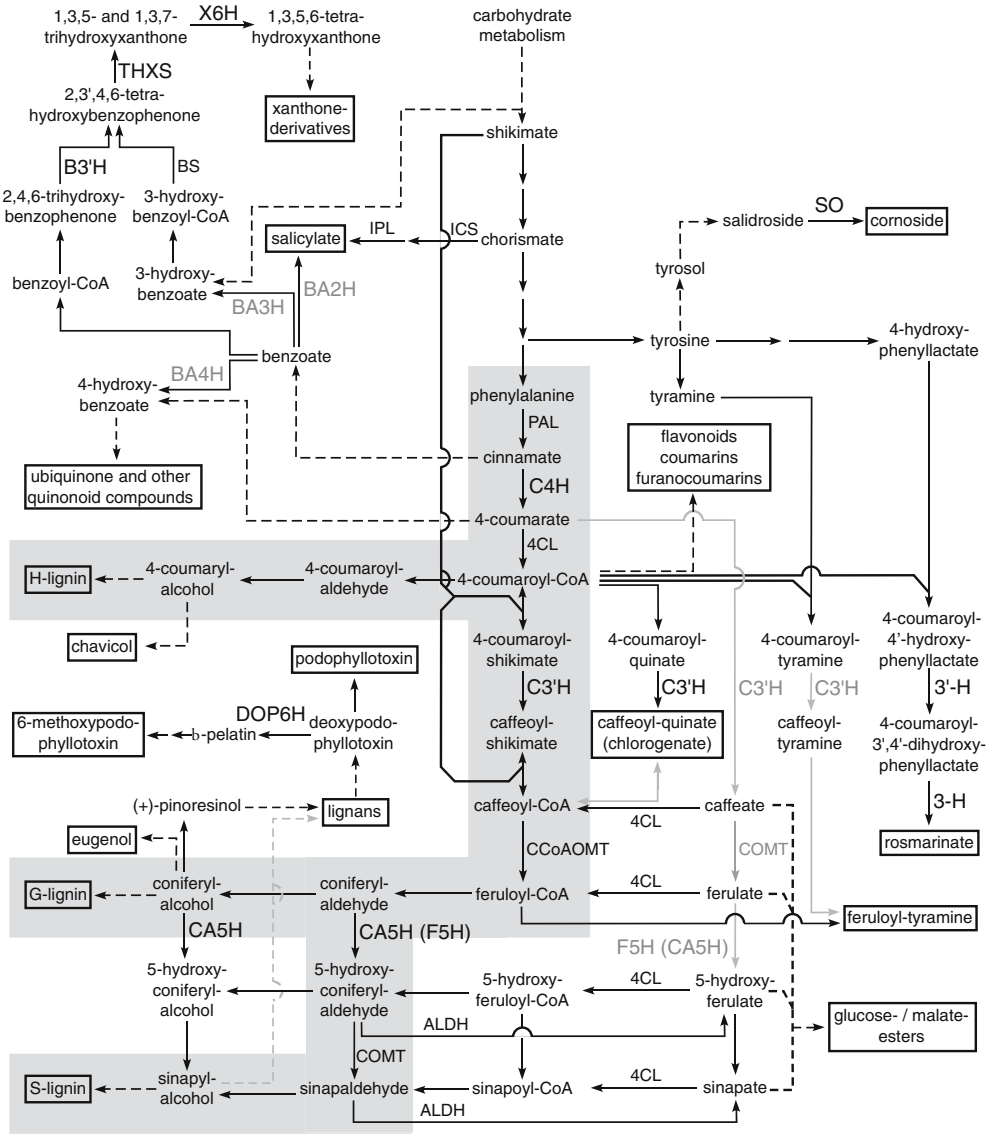


Fig. 1 Overview of the phenylpropanoid pathway. Shown are the metabolic routes of the phenylpropanoid pathway covered in this review. Names of cytochromes P450 involved are abbreviated beside and highlighted in bold print. Grey print indicates that an involvement is yet unclear or unlikely due to characterized biochemical properties. Multiple reaction steps are indicated by dashed lines. Selected other enzymes mentioned in the text are given in plain print. Abbreviations: 3′-H: 4-coumaroyl-4′-hydroxy-phenyllactate 3′-hydroxylase; 3-H: 4-coumaroyl-3′,4′-dihydroxy-phenyllactate 3-hydroxylase; 4CL: 4-coumarate:CoA ligase; ALDH: sinapaldehyde/coniferaldehyde dehydrogenase; B3′H: benzophenone 3′-hydroxylase; BA2H: benzoate 2-hydroxylase; BA3H: benzoate 3-hydroxylase; BA4H: benzoate 4-hydroxylase; BS: benzophenone synthase; C3′H: coumaroyl-shikimate/quinate 3′-hydroxylase; C4H: cinnamate 4-hydroxylase; CA5H (F5H): coniferaldehyde 5-hydroxylase (ferulate 5-hydroxylase); CCoAOMT: caffeoyl-CoA *O*-methyltransferase; COMT: 5-hydroxy-coniferaldehyde *O*-methyltransferase; DOP6H: deoxypodophyllotoxin 6-hydroxylase; ICS: isochorismate synthase; IPL: isochorismate pyruvate lyase; PAL: phenylalanine ammonia lyase; SO: salidroside oxidase; THXS: trihydrxyxanthone synthase; X6H: xanthone 6-hydroxylase

pathway are catalyzed by P450s. Here, we will describe recent advances in the molecular biology, biochemistry and genetics of these three hydroxylases encoded by divergent, plant specific, P450 families.

Cinnamate 4-hydroxylase (C4H, CYP73)

Biochemistry

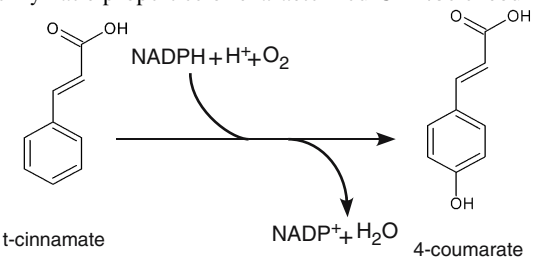
C4H has long been identified in plants and it was shown early that the reaction is catalyzed by a typical P450. Indeed, C4H was one of the first P450s to be characterized in plants (Nair and Vining, 1965; Russel and Conn 1967). Subsequently, C4H has been biochemically characterized in various plants and shown to be highly specific for its substrate *trans*-cinnamic acid with K_M values ranging from 2 to 30 μM. Other structurally closely related substances are not or only very poorly converted, and *trans*-conformation, side chain characteristics, and the presence of the carboxy group were essential for efficient catalysis (Werck-Reichhart 1995 and references therein).

In 1993, cDNAs encoding C4H were isolated from mung bean (*Vigna radiata, CYP73A2*), Jerusalem artichoke (*Helianthus tuberosus, CYP73A1*), and alfalfa (*Medicago sativa, CYP73A3*). The mung bean cDNA was isolated via PCR using degenerate primers based on peptide sequences derived from purified C4H (Mizutani et al. 1993), the Jerusalem artichoke cDNA via screening of an expression library using a specific antibody raised against the purified C4H (Teutsch et al. 1993; Werck-Reichhart et al. 1993), and the alfalfa clone by screening an expression library with an antibody raised against a divergent CYP71 from avocado (Fahrendorf and Dixon 1993). While expression of the alfalfa cDNA (*CYP73A3*) in an unmodified yeast strain resulted in low but clearly detectable amounts of C4H activity, expression of *CYP73A1* in yeast cells over-expressing either the yeast or human cytochrome P450 reductase yielded highly active C4H, characterized by a high turnover rate and efficiency for cinnamate conversion (Table 1).

Based on sequence information of these three C4Hs, homologous CYP73s were identified in numerous species by means of library screening, PCR with degenerate primers, and data mining of large scale cDNA and genome sequencing projects (Fig. 2). While most assignments are based solely on sequence similarity, some cDNAs were used to determine enzymatic properties of the respective recombinant proteins, mostly by expression in modified yeast cells over-expressing a P450 reductase (Table 1 and references therein). These studies showed that all CYP73s analyzed catalyze cinnamate 4-hydroxylation with high efficiency (K_M values ranging from 1.0 to 8.9 μM). Turnover rates or specific activities varied tremendously, but this is more likely due to differences in the expression system employed, rather than reflecting true differences in enzymatic properties.

CYP73s are the best characterized plant P450s from an enzymological point of view. They were shown to be highly specific to conversion of cinnamate. No activity was found with other phenolics such as 4-coumarate, ferulate, phenylalanine, benzoate, 3- or 4-hydroxybenzoate, phenylacetate, 2- or 3-hydroxy phenylacetate, or salicylate (Fahrendorf and Dixon 1993; Pierrel et al. 1994; Overkamp et al. 2000; Hübner et al. 2003). No hydroxylation activity was found with structurally unrelated natural compounds, which are targets of P450 action, such as terpenoids, fatty acids, coumarins, or furanocoumarins (Pierrel et al. 1994). However, CYP73A1 is capable, albeit with poor efficiency, of dealkylating the naturally occurring 7-methoxycoumarin (herniarin) and the xenobiotics 7-ethoxycoumarin, *p*-chloro-*N*-methylaniline, and of ring-methyl hydroxylating the herbicide chlorotoluron (Pierrel et al. 1994). Potent inhibitors, both competitive and irreversible, so called 'suicide' or mechanism based inhibitors, have been designed that specifically inactivate C4H both in vitro and in vivo. They lead to a drastic over-accumulation of salicylate in fungal elicitor-treated tobacco cells (Schoch et al. 2002). Fouranocoumarins are known inhibitors of mammalian P450 enzymes. Recently psoralen and methoxypsoralens were found to be potent mechanism based inhibitors of CYP73A1 from Jerusalem artichoke, a plant that

Table 1 Reaction catalyzed and enzymatic properties of characterized CYP73s encoding cinnamate 4-hydroxylase (C4H)



Name	K_M (μM)	k_{cat} (min^{-1})	V_{max} (pkat mg^{-1})	Reference
CYP73A3 (*Ms*)	–	–	0.3[a]	Fahrendorf and Dixon (1993)
CYP73A1 (*Ht*)	3.9	400	–	Urban et al. (1994)
CYP73A1 (*Cr*)	–	–	7[a]	Hotze et al. (1995)
CYP73A5 (*At*)	8.0	470	–	Urban et al. (1997)
CYP73A11 (*Gm*)	1.3	–	197	Schopfer and Ebel (1998)
CYP73A15 (*Pv*)	6.4	168	–	Nedelkina et al. (1999)
CYP73A10 (*Pc*)	5.0	–	1000	Koopmann et al. (1999)
CYP73A17 (*Ta*)	–	–	148[a]	Batard et al. (2000)
CYP73A9 (*Ca*)	–	–	Active[b]	Overkamp et al. (2000)
CYP73A (*Ptd*)	–	274[a]	–	Ro et al. (2001)
CYP73A41 (*Am*)	8.9	–	2.5	Hübner et al. (2003)
CYP73A32 (*Rg*)	1.0	162	–	Gravot et al. (2004)

Species abbreviations. *At*: *Arabidopsis thaliana*; *Am*: *Ammi majus* (bishop.s weed); *Ca*: *Cicer arietinum* (chickpea); *Cr*: *Catharanthus roseus*; *Gm*: *Glycine max* (soybean); *Ht*: *Helianthus tuberosus* (Jerusalem artichoke); *Ms*: *Medicago sativa* (alfalfa); *Pc*: *Petroselinum crispum* (parsley); *Pv*: *Phaseolus vulgaris* (French bean); *Ptd*: *Populus trichocarpa* × *deltoides* (hybrid poplar); *Rg*: *Ruta graveolens* (common rue); *Ta*: *Triticum aestivum* (wheat)

[a] Only specific activities were reported, these were given instead of k_{cat} or V_{max}. Note that activity values are derived from divergent expression systems and thus likely reflect technical rather than enzymatic differences

[b] No specific activities were reported

does not produce these furanocoumarins. In contrast, CYP73A32 from *Ruta graveolens* and CYP73A10 from parsley (*Petroselinum crispum*), both of which accumulate furanocoumarins, were less affected by these suicide inhibitors, which suggests an evolutionary adaptation to tolerate toxic metabolites in plants that produce the very compounds (Gravot et al. 2004).

Phylogeny

The majority of cDNA sequences encoding CYP73As available to date are highly similar to each other and they form a cluster of closely related sequences in phylogenetic reconstructions (termed class I in Fig. 2). These sequences contain a classical N-terminal targeting signal, and it was shown that C4H is indeed targeted to the endoplasmatic reticulum (ER) (Ro et al. 2001; Achnine et al. 2004). Most biochemically characterized proteins fall into this cluster (Table 1 and references therein). A divergent clade (class II) of CYP73s encompasses two branches. One of them (class IIA) includes typical CYP73s isolated from monocots, for example the wheat (*Triticum aestivum*) CYP73A17 with confirmed C4H activity (Batard et al. 2000). The second branch (termed class IIB) is illustrated by CYP73A15 from French bean (*Phaseolus vulgaris*) (Nedelkina et al. 1999). These class IIB sequences show a low overall sequence similarity to previously identified C4Hs from dicots or monocots, and an insertion of three amino acids in the meander region (Hasemann et al. 1995) upstream of the heme-binding cysteine. Such an insertion is expected to have an significant impact on the catalytic site structure. This group of CYP73s is also characterized by an atypical serine/threonine-rich N-terminal sequence that is possibly indicative of a non ER subcellular localization.

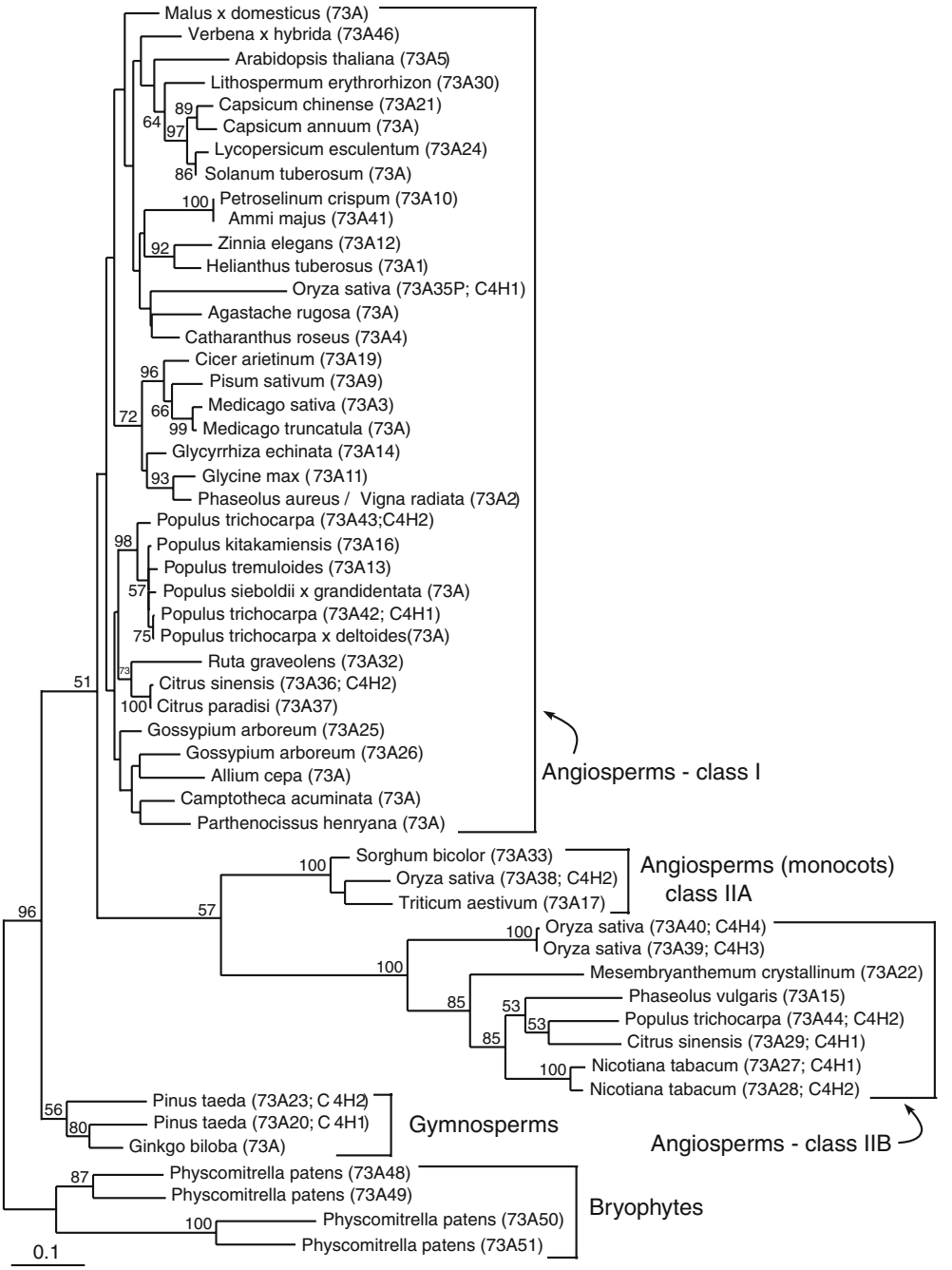


Fig. 2 Phylogenetic reconstruction of the CYP73A (C4H) family. An alignment of full length protein sequences was generated using Dialign (Morgenstern 2004) and unequivocally aligned regions were used for a maximum likelihood analysis using Phylip (Felsenstein 2005). Bootsrap values from 100 replicates higher than 50 are given at the nodes. When more divergent (CYP98) sequences were included, the CYP73s from *Physcomitrella* were found at the base of the CYP73 clade and were thus used to root this tree

However, CYP75A15 expressed in yeast was shown to encode a bona fide C4H (Table 1), although its efficient expression in yeast required replacement of the N-terminal region with the ER anchor from CYP73A1 (Batard et al. 2000). The French bean CYP73A15 was the first class II

C4H characterized, but additional members of this divergent phylogenetic clade (Fig. 2) have been characterized in orange (*Citrus sinensis*; 73A29/C4H2; Betz et al. 2001), and are found in tobacco (*Nicotiana tabacum*, CYP73A27 and 28; Ralston et al. 2001), ice plant (*Mesembryanthemum crystallinum*, CYP73A22; GenBank Accession AAD11427), poplar (CYP73A44/C4H2; Joint Genomics Institute, *Populus trichocarpa* v. 1.0; http://genome.jgi-psf.org/Poptr1/Poptr1.home.html) and rice genomes (International Rice Genome Project 2005).

While canonical class I CYP73s are usually not duplicated, two copies of the class II CYP73 are present in tobacco and rice genomes. Class II C4Hs from angiosperms are different from class I proteins with sequence similarities ranging from 58% to 63%, but are also more divergent among each other (72–95% identity) than class I proteins, which have identities ranging from 82% to 99% on the deduced amino acid level. It appears that a gene duplication prior to the divergence of monocots and dicots led to the divergent isoforms in angiosperms. Interestingly, independent duplications are also present in Bryophytes and Gymnosperms. While monocots predominantly maintained the class II enzymes (all monocot sequences are of class II, except a likely pseudogene of class I present in the rice genome (International Rice Genome Project, 2005), the predominant isoforms of dicots appears to be of class I. Some dicots however have lost their class II protein, since C4H is encoded by single copy genes in many species based on genomic DNA blot analysis (e.g., Mizutani et al. 1997; Koopmann et al. 1999; Hübner et al. 2003), and the complete sequence of the Arabidopsis genome revealed no class II gene (Raes et al. 2003). In reverse, all angiosperms shown to posses a class II isoform also appear to contain a class I counterpart (Fig. 2 and Betz et al. 2001; Nedelkina et al. 1999). In addition to the full length sequences available from *Citrus, Populus*, and *Phaseolus* (Fig. 2), expressed sequence tags (ESTs) for both classes are also available from tobacco (*Nicotiana tabacum*), potato (*Solanum tuberosum*), basil (*Ocimum basilicum*), and cotton (*Gossypium hirsutum*) proving that both classes can exist in the same species. Taken together, the class I in dicots and class IIA in monocots thus seem to be the most critical for the plant, respectively, but both the evolutionary divergence and the differences in wound induced expression of the two orange C4Hs (Betz et al. 2001) suggest that different physiological functions were probably acquired in plants which maintained the duplication. However, these functions still need to be established. When used for anti-sense constructions in transgenic tobacco, *C4H* suppression using either a class I or a class II gene resulted in reduced lignin deposition (see below for detail).

CYP73 sequences from gymnosperms group at the base of both angiosperm clusters, but are more similar to class I sequences (~78% identical on amino acid level) than to class II proteins (ranging from 59% to 72%). C4H activity is also present in the hornwort *Anthoceros agrestis* that accumulates rosmarinic acid (Petersen 2003) and four CYP73 homologues have been identified in ESTs of the moss *Physcomitrella patens* (Lang et al. 2005), which form the most divergent cluster in phylogenetic reconstructions (Fig. 2), but none of the gymnosperm and bryophyte proteins have been biochemically characterized.

Regulation

Numerous studies have characterized *C4H* expression in divergent species, and, while *C4H* expression is detectable in most organs analyzed, it is predominantly expressed in organs with high phenylpropanoid biosynthesis and/or undergoing lignification. *Zinnia elegans CYP73A12* transcript levels are elevated during trans-differentiation of mesophyll cells to tracheary elements, which is correlated with C4H activity and lignin deposition. *CYP73A12* is also predominantly expressed in differentiating xylem and phloem fibers based on tissue print hybridization (Ye 1996). The *Arabidopsis* and pea *C4H* genes are mainly expressed in stems and roots (Frank et al. 1996; Mizutani et al. 1997; Urban et al. 1997) and a coordinated expression of *Arabidopsis C4H* with all other genes of the phenylpropanoid pathway is evident during development of inflorescence stems (Ehlting et al. 2005). GUS reporter gene expression driven by the *Arabidopsis C4H*

promoter, was found predominantly in roots, leaf veins, vascular bundles and in interfascicular fibres (Bell-Lelong et al. 1997). Interestingly, *Arabidopsis C4H* expression was shown to oscillate in a diurnal fashion in leaves, in tight coordination with other genes of the pathway (Rogers et al. 2005; Abdulrazzak et al, 2006). Rather unexpectedly, the class II *C4H* from French bean was also associated to lignification, and shown to be co-expressed with *PAL* in xylogenic induction media (Nedelkina et al. 1999).

High *C4H* expression was also reported in correlation with the production of defense compounds. In parsley, transcript and protein showed highest abundance in flowers and pedicels and, based on in situ analyses, accumulate mainly in epidermal and oilduct epithelial cells, vascular tissue, and major parts of the ovule, all sites known for high phenylpropanoid biosynthetic activity and production of furanocoumarins (Koopmann and Hahlbrock 1997; Koopmann et al. 1999). The parsley gene is also induced locally at infection sites with *Phytophtora sojae* based on in situ hybridization (Koopmann et al. 1999), and fungal elicitors lead to increased expression in leaves and to a biphasic expression in cell cultures. A similar expression profile was described for *PAL* and *4CL* (Logemann et al. 1995; Batz et al. 1998). Coordinated induction with genes involved in isoflavonoid phytoalexin biosynthesis by elicitors was also observed for the alfalfa *C4H* (*CYP73A3*; Ni et al. 1996). Fungal elicitor induced expression in cell cultures was also shown for C4H from Slash pine (*Pinus elliottii*), *Catharantus roseus* (*CYP73A4*), soybean (*Glycine max, CYP73A11*), chickpea (*Cicer arietinum, CYP73A9*), and poplar (*Populus trichocarpa* × *deltoides*) (Mason and Davis 1997; Hotze et al. 1995; Schopfer and Ebel 1998; Overkamp et al. 2000; Ro et al. 2001). The class II *C4H* from French bean was transiently induced upon elicitor treatment, while *PAL* transcript level remains high over the time period analyzed (Nedelkina et al. 1999).

Likewise, *C4H* is coordinately induced by wounding as shown for the isoforms from parsley, *Arabidopsis*, pea, and Jerusalem artichoke (Logemann et al. 1995; Mizutani et al. 1997; Urban et al. 1997; Frank et al. 1996; Batard et al. 1997), and other environmental cues such as UV containing light and nutrient deprivation (Koopmann and Hahlbrock 1997; Urban et al. 1997; Hotze et al. 1995). *C4H* expression thus appears to be tightly coordinated with that of other genes involved in the biosynthesis of phenylpropanoid in demand, under all conditions analyzed. Moreover, transcript accumulation was closely correlated with protein abundance and enzymatic activity, which strongly suggests a primary regulation at the transcriptional level (Batard et al. 1997; Koopmann et al. 1999; Ye 1996).

Indeed, upstream regions of *C4H* genes do contain putative *cis*-acting elements known from other phenylpropanoid genes further supporting a coordinated transcriptional regulation of the phenylpropanoid pathway. The *Arabidopsis C4H* promoters contains P, L, and A boxes previously identified as in vivo footprints in the parsley *PAL* promoter (Bell-Lelong et al. 1997). The pea *C4H* promoter contains, in addition, a Box IV element, an AT rich element identified in elicitor induced footprinting of the pea *PAL* promoter, and a PMYB consensus sequence (Whitbred and Schuler 2000). The P and L boxes, which constitute in their core AC rich elements, have been shown to be target sites of MYB transcription factors: a number of MYB factors bind to these elements and can *trans*-activate the expression from promoters containing them (Rogers and Campbell 2004 and references therein). Several divergent MYB transcription factors have been shown to be involved in regulating lignin biosynthesis and phenylpropanoid gene expression in woody species (Karpinska et al. 2004; Patzlaff et al. 2003; Goicoechea et al. 2005), however, without affecting *C4H* gene expression. In contrast, overexpression of the snapdragon (*Antirrhinum majus*) *MYB308* gene in tobacco resulted in reduced *C4H* expression, reduced *4CL* and *CAD* (but not *PAL*), and reduced G/S lignin and chlorogenic acid (Tamagnone et al. 1998). In addition, an *Arabidopsis* T-DNA insertion mutant of *AtMYB4*, a likely ortholog of *AmMYB308*, was characterized by higher levels of sinapoyl malate (and lower levels in overexpression lines) resulting in higher tolerance of

myb4 plants to UV-B irradiation (and reduced tolerance when over-expressed). In *myb4* null mutants, only C4H expression is increased, while *CCoAOMT* is decreased and other phenylpropanoid genes are not affected (Jin et al. 2000). Based on these, and other expression data, it appears that MYB4 from *Arabidopsis* acts as negative regulator of sinapate ester biosynthesis mainly by repressing *C4H* expression. *MYB4* transcripts are repressed by UV and wounding, which results in activated *C4H* expression. Higher doses of the repressor seem to be needed for the inactivation of other genes of the phenylpropanoid pathway (Jin et al. 2000). C4H may thus constitute a rate-limiting step for channeling carbon flux into the pathway. This is supported by the analysis of Anterola et al. (2002): Based on the lack of induced expression in suspension cells of *Pinus taeda* supplemented with phenylalanine, a treatment that does induce expression of other phenylpropanoid genes, and critical evaluation of the literature, they concluded that the activity of C4H and phenylalanine availability constitute the rate limiting factors controlling carbon allocation to the pathway, while amounts of PAL and other enzymes of the pathway are modulated by demand. This interpretation is further corroborated by studies of transgenic tobacco with reduced C4H activity. Sense or anti-sense suppression using the alfalfa *CYP73A3* cDNA under control of the *35S* promoter result in a clear reduction of PAL activity in leaves and stems, and it appears that *C4H* down-regulation can reduce PAL activity (and transcription) even if *PAL* is being expressed from an artificially enhanced promoter (Blount et al. 2000). Using a reverse experimental setup, there was no effect of down-regulation of *PAL* on C4H activity, which supports a model where PAL is regulated by negative feedback control exerted at the level of C4H activity (Blount et al. 2000).

Using the same transgenic lines, it was also shown that *C4H* down-regulation results in lower levels of chlorogenic acid and other soluble caffeic acid esters, but over-expression of *C4H* in leaves does not consistently result in increased accumulation of chlorogenic acid (Blount et al. 2000). A cell culture derived from over-expression lines displayed few metabolic phenotypes. Upon treatment with a fungal elicitor, accumulation of most phenylpropanoids (including chlorogenic acid) remained unaltered, but higher levels of acetosyringone, a likely derivative of sinapate, was found to over-accumulate (Blount et al. 2002). Likewise, over-expression of the alfalfa *C4H* in tobacco, resulting in a twofold activity increase, had no effect on lignin composition, while down-regulation (more than twofold lower activity) resulted in reduced total Klason lignin (Sewalt et al. 1997). Surprisingly, a greater impact on syringyl units was observed. A drastic decrease in S residues was accompanied by a smaller decrease in G residues, resulting in strongly reduced S/G ratios. In contrast, *PAL* down-regulation, which also led to decreased total lignin, increased the S/G ratio. Sewalt et al. (1997) thus suggested that down-regulation of *PAL* or *C4H* could lead to differential feed-forward effects on later downstream enzymes or, less likely, that parallel pathways exist (maybe via metabolic channeling).

Only EST class I sequences, the likely target of the alfalfa C4H, are available from tobacco, but two full length class II sequences were isolated (Ralston et al. 2001). Those are the likely targets of suppression by anti-sense or sense expression of the French bean *CYP73A15* in tobacco. Such experiments led to reduced C4H protein, activity (down to 10% of wild type levels), and to reduced lignin staining in young tobacco plants (Blee et al. 2001). Mature plants displayed delayed accumulation of lignin and were characterized by a slightly reduced amount of total lignin, with decreased S-proportion comparable to that found using the class I alfalfa *C4H* for suppression (Blee et al. 2001). The *Medicago truncatula* class I *C4H* cDNA under control of the vascular tissue-specific bean *PAL2* promoter was also used for anti-sense suppression in transgenic alfalfa (*Medicago sativa*). This resulted in a reduction in C4H activity down to 21% of wild-type (Reddy et al. 2005) concomitant with a more than sevenfold decrease in lignin content, which was almost exclusively due to a reduction in S-units. This *C4H* suppression led to drastically increased in vitro and in situ digestibility of stem or total

forage. A loss of purple-blue flower pigmentation was also observed (Reddy et al. 2005). Together, these results clearly show that both class I and class II C4Hs can interfere with the biosynthesis of lignin and other phenylpropanoids, but also consistently show that syringyl derived lignin is predominantly affected, which comes as a surprise as C4H is expected to be equally involved in G- and S-units. This puzzle is so far unresolved.

In all experiments described so far, it must however be kept in mind that the use of exogenous genes in transgenic studies and the only partial reduction using sense or anti-sense approaches may complicate interpretations. It is interesting to note that to date no studies describing null-mutants are available. The only *C4H* mutants described are the EMS *ref3* mutants of *Arabidopsis* (note in Raes et al. 2003). The *ref* mutants were identified based on their *reduced epidermal fluorescence* phenotype caused by a lack or reduction of sinapoyl malate in leaves (Ruegger and Chapple 2001). The *ref3* mutants displays a severely dwarfed morphology, increased branching, and strong alleles are male sterile. It is characterized by a severe reduction of all sinapate esters, both in leaves and seeds. *CYP73A5* inactivation leads to a strong reduction of lignin content based on thioglycolic acid derivatization (~ fourfold in the strong *ref3-2* allele). It has however little effect on the S/G ratio determined by alkaline nitrobenzene oxidation. Moreover, *ref3* seeds are pale, and stems do not show the typical purple coloration due to the accumulation of (pro-) anthocyanin, which indicates a suppression of flavonoid biosynthesis (Ruegger and Chapple 2001). These metabolic phenotypes are fully consistent with, and proof of a physiological function of *REF3* (*CYP73A5*) at the entry point of the phenylpropanoid metabolism. The strong morphological impact of the *ref3* mutations, which might not even represent null-alleles, already suggests that either deleterious substances accumulate, or that downstream compounds needed for normal development are missing in the mutant (Ruegger and Chapple 2001). A similar observation was made in studies related to null mutants of C3′H.

Coumaroyl-shikimate 3′-hydroxylase (C3′H, CYP98)

An elusive step in the phenolic metabolism

The mechanism of the 3-hydroxylation (or *meta*-hydroxylation) of phenolic precursors has long remained elusive. *Meta*-hydroxylation is however needed for the biosynthesis of guaiacyl and syringyl units of lignin, of UV absorbing pigments such as sinapoyl malate and sinapoyl choline, and also for the formation of many antioxidants and bioactive esters or amides (such as chlorogenic acid, rosmarinic acid and caffeoyltyramine), of coumarins (such as scopoletin), flavors, and fragrances (such as vanillin, gingerol, capsaicin, safrole and eugenol).

Originally, it was believed that the 3-hydroxylation occurs on the free acids, prior to conjugation and/or reduction. The enzymes catalyzing this reaction, however, were never characterized in detail. They were described as ascorbate-, NADPH- or FAD-dependent oxygenases (Vaughan and Butt 1970; Stafford and Dresler 1972; Boniwell and Butt 1986; Kojima and Takeuchi 1989), as a plastidic enzyme using plastoquinone or ferredoxin as electron donor (Bartlett et al. 1972), or as a non-specific phenolase that also oxidizes dihydroxyphenols to orthoquinones (Vaughan and Butt 1970). In animals, 3-hydroxylation of tyrosine (as well as hydroxylation of phenylalanine and tryptophane) are catalyzed by a the family of tetrahydropterin-dependent aromatic amino acid hydroxylases that use molecular oxygen and are iron containing enzymes (Fitzpatrick 2003), but no plant homologs appear to exist.

More recently, an involvement of ester intermediates was suggested, with three enzymes being described to catalyze the 3-hydroxylation of 4-coumaroyl-CoA: a non-specific polyphenol oxidase, a soluble FAD-dependent hydroxylase, and a Zn^{2+}-dependent dioxygenase that was described to be inactive at cytoplasmic pH (Kamsteeg et al. 1981; Kneusel et al. 1989; Wang et al. 1997). An involvement of P450 enzymes was also suggested for the *meta*-hydroxylation of quinate and shikimate esters of *p*-coumaric acid, which lead to the synthesis of chlorogenic acid

and caffeoyl-shikimate repectively (Heller and Kühnl 1985; Kühnl et al. 1987). None of these enzymes however was characterized at the molecular level, nor was a contribution to the biosynthesis of lignin monomers indicated.

An unexpected route revealed by genomic approaches

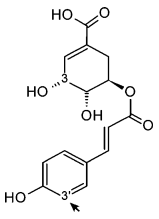
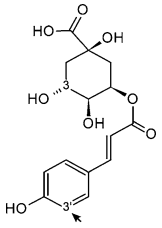
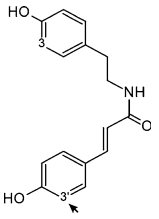
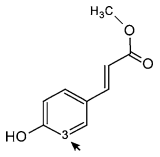
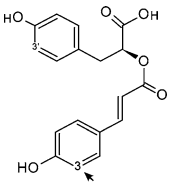
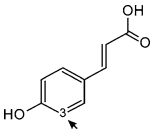
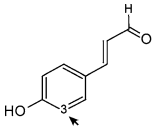
Only in the last years three groups independently identified the CYP98 family of cytochrome P450 enzymes as the major 3-hydroxylase in the phenylpropanoid pathway using either bioinformatic or genetic approaches (Schoch et al. 2001; Nair et al. 2002; Franke et al. 2002a). Based on sequence similarity to *C4H* (*CYP73A5*), on similar expression patterns, on the presence of conserved putative *cis*-acting elements, and the existence of putative orthologous ESTs in diverse species, *CYP98A3* was selected as a candidate by Schoch et al. (2001) and Nair et al. (2002). In contrast, Franke et al. (2002a, b) identified *CYP98A3* via positional cloning of the *reduced epidermal fluorescence 8* (*ref8*) mutant, which is deficient in sinapate esters and which is also unable to synthesize caffeic acid. The P450 protein inactivation in the *ref8* mutant resulted from a missense point mutation adjacent to the region coding for the heme-binding site of the P450 protein. Expression profiling based on RNA blot analysis, immuno-localization and promoter-GUS lines showed expression predominantly in lignifying tissues, resembling known phenylpropanoid genes like *C4H*. However, using promoter-GUS fusions a somewhat more restricted tissue specific expression of *CYP98A3* compared to *C4H* was observed; e.g., *CYP98A3* is not expressed in seeds and does not show extensive expression in flower tissues, but promoter activity is restricted to vascular tissues. As discussed by Nair et al. (2002), this restricted expression pattern however needs to be interpreted carefully as additional *cis*-acting element might be missing in the *CYP98A3* promoter fragment used.

All three groups used recombinant protein expressed in yeast for the characterization of enzymatic properties of CYP98A3 (Table 2). This showed that 4-coumaric acid, 4-coumaroyl-CoA, 4-coumaroyl-glucose, as well as the corresponding aldehyde and alcohol were poorly or not metabolized by CYP98A3 (Schoch et al. 2001; Nair et al. 2002; Franke et al. 2002a). Higher activity was observed with 4-coumaroyl methyl ester, but the kinetic properties for this substrate were still poor compared to other enzymes of the phenylpropanoid pathway (Franke et al. 2002a). However, exploration of further potential substrates, partially driven by reports of Heller and Kühnl (1985) and of Kühnl et al. (1987), led to the demonstration that the shikimate and quinate esters of 4-coumaric acid are very actively hydroxylated in the *meta* position on the phenolic ring (Schoch et al. 2001). The turnover and catalytic efficiency of this reaction was higher than the *para*-hydroxylation of cinnamic acid by C4H, with shikimate ester being the best substrate. CYP98A3 converts exclusively the *trans* isomers, and metabolizes the naturally abundant 5-*O*-*p*-coumaroyl isomers with significantly higher efficiency than the 4-*O*- and 3-*O*-isomers of shikimate and quinate (Schoch et al. 2001). CYP98A3 was thus confirmed as a *meta*-hydroxylase of the phenolic ring functioning as a 5-*O*-(4-coumaroyl)shikimate/quinat- 3′-hydroxylase (C3′H). It is noteworthy that thus C3′H also catalyzes the final step of the biosynthesis of chlorogenic acid (caffeoyl-quinate), a widespread antioxidant and quantitatively major phenolic in many plants (Fig. 1).

Multifunctional enzymes?

Simultaneously to the described work in *Arabidopsis*, a search for candidate genes potentially involved in the biosynthesis of eugenol, a coniferylalcohol-derived compound in sweet basil (*Ocimum basilicum*), led to the isolation of two *CYP98A13* variants from peltate glands (Gang et al. 2002). When expressed in yeast, CYP98A13s were shown to catalyze the *meta*-hydroxylation of shikimate and quinate esters in a similar way to CYP98A3 from *Arabidopsis*, while 4-coumarate and 4-coumaroyl-CoA were very poor substrates (Table 2). Like other Lamiaceae, sweet basil synthesizes rosmarinic acid and the phenyllactic ester of 4-coumaric acid, the precursor of rosmarinic acid (Fig. 1), was thus also assayed as a substrate of CYP98A13. The

Table 2 Substrates and enzymatic properties of characterized CYP98s encoding coumaroylshikimate 3′-hydroxylase

	 4-coumaroyl-shikimate			 4-coumaroyl-quinate			 4-coumaroyl-tyramin			 4-coumaroyl methyl ester			 4-coumaroyl-4′-hydroxy-phenyllactate			 4-coumarate			 4-coumaraldehyde		
	K_M[a]	k_{cat}	k_{cat}/K_M	K_M	k_{cat}	k_{cat}/K_M	K_M	k_{cat}	k_{cat}/K_M	K_M	k_{cat}	k_{cat}/K_M	K_M	k_{cat}	k_{cat}/K_M	K_M	k_{cat}	k_{cat}/K_M	K_M	k_{cat}	k_{cat}/K_M
CYP98A3 (*At*)	7	612	87[b]	18	399	22[b]	Low activity[c]			2,500	0.05	< 0.01[d]	No activity[c]			Very low activity[d,e]			Very low activity[d]		
CYP98A13v1 (*Ob*)	3	109	41[f]	28	91	3[f]	nd[g]				nd		nd			5,100	nd	nd[f]	nd		
CYP98A13v2 (*Ob*)	3	163	56[f]	18	189	10[f]	Medium activity[c]				nd		Low activity[c]			5,100	nd	nd[f]	nd		
CYP98A10 (*Ts*)	49	546	11[c]	97	46	0.5[c]	nd	< 0.5	nd[c]		nd		No activity[c]			No activity[c]			nd		
CYP98A11 (*Ts*)	7	1,024	185[c]	6	410	68[c]	140	25	0.2[c]		nd		No activity[c]			Very low activity[c]			nd		
CYP98A12 (*Ts*)	7	204	31[c]	4	70	17[c]	53	13	0.2[c]		nd		No activity[c]			Very low activity[c]			nd		
CYP98A6 (*Le*)		nd			nd			nd			nd		Activity[h]			nd			nd		

At: *Arabidopsis thaliana*; *Ob*: *Ocimum basilicum* (sweet basil); *Ts*: *Triticum sativum* (wheat); *Le*: *Lithospermum erythrorhizon*

[a] *Units*. K_M (μM); k_{cat} (min^{-1}); k_{cat}/K_M (min^{-1} μM^{-1})

[b] Schoch et al. (2001)

[c] Morant et al. (2006)

[d] Franke et al. (2002a)

[e] Nair et al. (2002)

[f] Gang et al. (2002)

[g] Not determined

[h] Matsuno et al. (2002)

meta-hydroxylation of the phenyl moiety of this ester proceeded at a rate that was estimated to be about 15% of the metabolism of the shikimate ester, which casts some doubt on its physiological function in rosmarinic acid biosynthesis (Gang et al. 2002). However, another C3′H-encoding cDNA (*CYP98A6*) was isolated from a different rosmarinic acid producing plant, *Lithospermum erythrorhizon* (Boraginaceae). Expressed in yeast, the CYP98A6 protein was also shown to catalyze the 3-hydroxylation of 4-coumaroyl-4′-hydroxyphenyllactic acid, and was thus implicated in rosmarinic acid biosynthesis (Matsuno et al. 2002). In the latter case, kinetic properties were not determined in detail, and other 4-coumaroyl esters were not assayed. The most detailed biochemical characterization of the 3′- and 3-hydroxylases in the biosynthesis of rosmarinic acid were performed using microsomal fractions from cell cultures of *Coleus blumei*. Both hydroxylations were shown to rely on P450 enzymes which were found to introduce the corresponding hydroxyl groups into the aromatic rings of 4-coumaroyl-4′-hydroxyphenyllactate and 4-coumaroyl-3′,4′-dihydroxyphenyllactate, respectively, with high specificity and activity (Petersen 1997). Isolation of the P450s catalyzing these two hydroxylation steps in *C. blumei* is not yet reported. Their characterization might help to clarify the role of CYP98s in the 3′-hydroxylation reaction.

More recently, eight CYP98 genes were isolated from wheat (*Triticum aestivum*) based on a new PCR approach using very short conserved sequences (Morant et al. 2002). Seven of them contained complete open reading frames allowing successful expression in yeast. While four enzymes with significant sequence changes compared with canonical CYP98s were inactive with a variety of 4-coumaroyl esters, the remaining three others were active, with 4-coumaroyl-shikimate and 4-coumaroyl-quinate being the preferred substrates. However, differences in substrate specificities were indicative of divergent physiological functions (Morant et al. 2006). Interestingly, two of the wheat enzymes, CYP98A11 and CYP98A12, were capable of *meta*-hydroxylating 4-coumaroyl-tyramine with kinetic properties that might be indicative of a physiological function in feruloyl-tyramine biosynthesis (Fig. 1, Table 2), while the phenylactate ester, which is metabolized by CYP98s from rosmarinic acid accumulating plants, was not a substrate of the wheat enzymes. A CYP98A11 variant, CYP98A10, was also isolated, which showed almost complete loss of 4-coumaroyl-tyramine hydroxylase and strongly reduced 4-coumaroylquinate hydroxylase activity. Directed mutagenesis restoring a CYP98A11-like sequence simultaneously restored both activities (Morant et al. 2006). Efficient metabolism of quinate ester and tyramine amide of *p*-coumaric acid by CYP98s might thus be correlated. Feruloyl-tyramine is a common constituent of the cell wall, a building block of suberin (Facchini et al. 2000; Bernards and Lewis 1998), and is deposited around wounds or pathogen penetration sites. So far, feruloyl-tyramine was usually assumed to be formed via transacylation from feruloyl-CoA, and the activities of CYP98A11 and CYP98A12 with 4-coumaroyltyramine are much lower compared with the hydroxylation of the shikimate ester (Morant et al. 2006). The in vivo involvement of CYP98s in a direct route to ferruloyl-tyramine has thus to be confirmed.

In summary, the biochemical studies show that the 4-coumaroyl ester of shikimate is the preferred substrate of all the tested CYP98s, followed by the quinate ester and the enzyme is thus termed C3′H. However, additional 4-coumaroyl esters or amides can be *meta*-hydroxylated by CYP98s. Although the physiological significance of such side reactions needs further investigation, it is striking that only CYP98s from species known to accumulate a given compound have the capacities to hydroxylate the respective precursor, e.g., 4-coumaroyl-4′-hydroxyphenyllactate is only hydroxylated by CYP98A13 from sweet basil and by CYP98A6 from *Lithospermum* and both species are known to accumulate rosmarinic acid. Further studies will need to take into account the possible tissue- or cell-specific expression of dedicated hydroxycinnamoyl tranferases, channeling processes, or the local accumulation of specific precursors (e.g., in glandular trichomes).

Phylogeny

To date, an additional 12 *CYP98* full length sequences were deposited into Genbank, and the

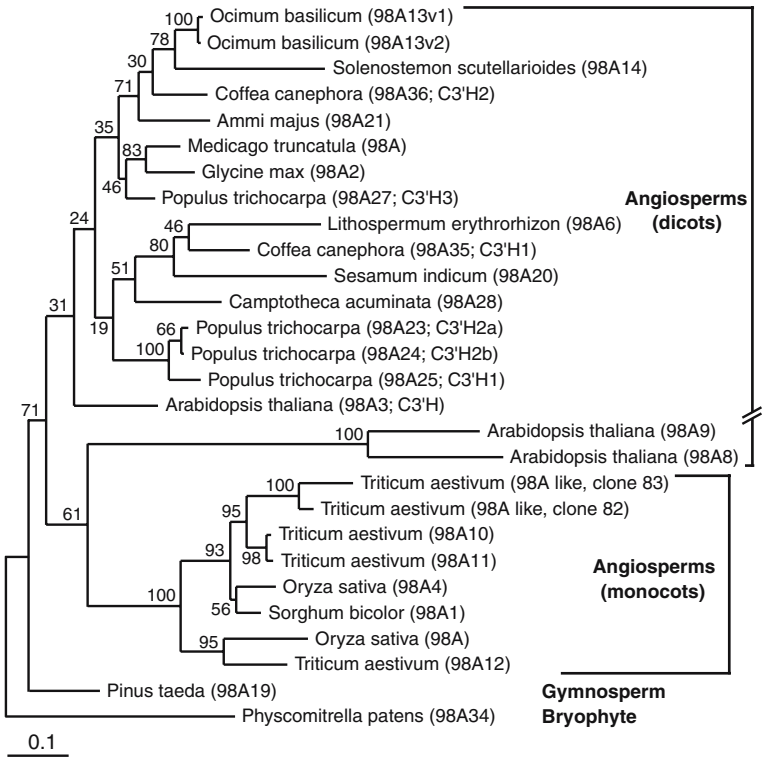


Fig. 3 Phylogenetic reconstruction of the CYP98A (C3′H) family. An alignment of full length protein sequences was generated using Dialign (Morgenstern 2004) and unequivocally aligned regions were used for a maximum likelihood analysis using Phylip (Felsenstein 2005). Bootsrap values from 100 replicates are given at the nodes. The *Physcomitrella* protein were used to root this tree

deduced protein sequences were used for phylogenetic reconstruction (Fig. 3). Two additional *CYP98s* are present in the *Arabidopsis* genome. They form a divergent clade and were shown not to encode C3′Hs (Schoch et al. 2001). No genes belonging to this clade are so far reported in other plant species. The rice (*Oryza sativa*) genome (International Rice Genome Sequencing Project 2005) contains a single putative *C3′H* and an additional likely pseudogene, which group with all other monocot sequences as a sister clade to the dicot sequences. In contrast, the third plant with its genome completely deciphered, poplar (Joint Genomics Institute, *Populus trichocarpa* v. 1.0; http://genome.jgi-psf.org/Poptr1/Poptr1.home.html), contains three putative *C3′H* isoforms assuming that *C3′H2a* and *C3′H2b* are allelic (Björn Hamberger, unpublished; Fig. 3). While *C3′H1* and *C3′H2* probablyare derived from recent duplications, *C3′H3* is more divergent, and is part of a second clade within the dicot sequences. Likewise, two isoforms from coffee (*Coffea canephora*) exist (P450 web page by D. Nelson; http://drnelson.utmem.edu/Cytochrome P450.html) with each isoform being placed in one of the two major clades within the dicot sequences (Fig. 3). This might be indicative of a functional diversification of CYP98 enzymes within the dicot lineage. A similar diversification might have occurred in monocots, as indicated by the clustering of the rice pseudogene with the wheat *CYP98A12*. The sole sequence from a gymnosperm, *CYP98A19* from *Pinus taeda* groups at the base of the angiosperm sequences when the tree was rooted using the *Physcomitrella patens CYP98A34*. None of these additional enzymes have been characterized in detail for catalytic activity yet, but the *Medicago trunculata* gene was used for down-regulation of the alfalfa (*Medicago sativa*) ortholog (see below), and the gene from *Camptotheca acuminata*, a native medicinal plant in China, was shown to be expressed mainly in stems (Pi et al. 2006).

Functions in planta

The first functional proof of an essential role of C3′H in the phenylpropanoid pathway came

from the analysis of the phenolic composition of the *Arabidopsis ref8* mutant caused by a point mutation in *CYP98A3* (Franke et al. 2002b). This analysis demonstrated that both soluble (sinapoyl-malate in leaves and sinapoyl-choline in seeds) and lignin-associated *meta*-hydroxylated G and S units are drastically reduced in the mutant. Instead, mutant plants accumulate 4-coumarate esters in leaves, and to some extent in seeds. The *ref8* plants also accumulate wall bound esters of 4-coumarate and 4-hydroxybenzaldehyde, and the lignin content was reduced to 20–40% of wild type levels. The mutant plants form a lignin in bolting stems which consists primarily in 4-coumarate-derived H-units, while G- and S-units are found only in trace amounts (Franke et al. 2002b). The *ref8* plants display additional phenotypes including collapsed vessels, a dwarfed statute, and enhanced susceptibility to fungal attack (Franke et al. 2002b). A major impact of *CYP98A3* on plant development was further confirmed by the analysis of a T-DNA insertion knock-out mutant (Abdulrazzak et al. 2006). The *cyp98A3* T-DNA mutant shows an extreme dwarf phenotype and rarely ever develops a fertile inflorescence stem, but instead is developmentally arrested without showing signs of senescence. Inhibition of development in *cyp98A3* plants is associated with reduced cell growth. Chemical complementation with caffeoyl-shikimate suggests that a missing downstream product of the phenylpropanoid pathway is, at least in part, responsible for the extreme pertubations in plant growth (Abdulrazzak et al. 2006). As observed with the *ref8* mutant, lignin composition in aerial parts of the T-DNA mutant consists mainly of H-units, with very reduced levels of G- and S-units. Decreased amounts of soluble *meta*-hydroxylated simple phenolics and their esters is paralleled by an accumulation of 4-coumaroyl-esters and flavonoids. Similarly, down-regulation of the alfalfa (*Medicago sativa*) *C3′H* gene via antisense suppression resulted in strongly reduced C3′H activity (down to 3% of wild type levels) and, in consequence, to similar changes in lignin composition, with reduced total yield, and a drastic increase in H-lignin units (Reddy et al. 2005). A slight delay in development (late flowering) was also observed. *C3′H* down-regulation led to an improved digestibility both in vitro and in situ, an important agronomic property of forage crops, that seems to result more from decrease in lignin content than modified composition (Reddy et al. 2005). A detailed analysis of the lignin properties in *C3′H* down-regulated alfalfa lines including a novel methodology based on nuclear magnetic resonance confirmed the prominent increase in H-relative to G- and S-lignin, but also identified differences in the interunit linkages, namely an abolishment of β-1-coupling products and a concomitant increase in dibenzodioxocin and β-5-phenylcoumaran (Ralph et al. 2006).

Another phenolic 3-hydroxylase?

These results suggest that CYP98 is the major, if not sole *meta*-hydroxylase in the phenylpropanoid metabolism. However and surprisingly, *Arabidopsis cyp98A3* T-DNA mutants and co-suppressed plants produce detectable levels of sinapoyl-malate, significant amounts of sinapoylated flavonoids, and display an ectopic lignification phenotype, especially in roots, with substantial amounts of G- and S-units in the null mutant (Abdulrazzak et al. 2006). This indicates that an alternative pathway exists, at least in *Arabidopsis*, which appears to be activated independently of the prevalent pathway, potentially under stress conditions. Activation of this alternative pathway cannot complement a defect in the main CYP98-dependent pathway in *Arabidopsis*. But it is noteworthy that some plants can incorporate externally fed sinapic acid into S-lignin (Yamauchi et al. 2003). In addition, the identification of 4CL isoforms specifically activating sinapate to the coenzyme A ester in diverse plant lineages (Lindermayr et al. 2002; Hamberger and Hahlbrock 2004; Hamada et al. 2004) point to a role of the free acid in phenylpropanoid metabolism, at least in some plants. Although no specific physiological role for the sole sinapate activating 4CL in *Arabidopsis* could be identified when analyzed under conditions when the prevalent pathway via C3′H is functional (Costa et al. 2005), a role in the proposed alternative pathway should not be prematurely excluded.

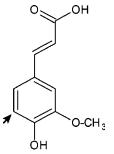
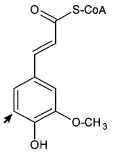
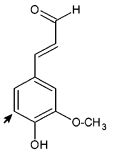
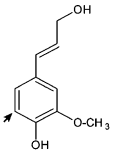
Coniferylaldehyde 5-hydroxylase (CA5H, CYP84)

An unexpected route revealed by genetics

The 5-hydroxylase of the phenylpropanoid metabolism was also identified by a genetic screen for *Arabidopsis* mutants lacking sinapoyl esters, in particular sinapoyl malate in leaves and sinapoyl choline in seeds (Chapple et al. 1992). As the mutant, originally called *sin1*, is almost devoid of S-lignin, accumulates ferulate, and can incorporate externally fed 5-hydroxyferulate (but not ferulate) into sinapate, an activity as ferulate 5-hydroxylase was suggested (Chapple et al. 1992). The mutant was later renamed *fah1*, for *ferulic acid hydroxylase*, when the gene was isolated from a T-DNA tagged allele by plasmid rescue (Meyer et al. 1996). The corresponding genomic region fused to the *35S* promoter did complement the *fah1* phenotype proving the successful identification of the gene. Sequence comparisons showed that *FAH1* (or *F5H* for ferulate 5-hydroxylase) encodes a cytochrome P450 and defines a new family, namely *CYP84* (Meyer et al. 1996). This finding was in agreement with a previous report on the partial characterization of ferulate 5-hydroxylase as a P450 in microsomal fractions from poplar (*Populus* × *euramericana*) stems, which was preferentially active in sclerenchyma cells rich in S-lignin (Grand 1984). This original report was however never confirmed in other plant species, and this is possibly due to the unexpected substrate specificity of the 5-hydroxylase. Indeed, using a *CYP84A4* cDNA from sweetgum (*Liquidambar styraciflua*) expressed in yeast, it was shown that the enzyme hydroxylates efficiently coniferylaldehyde (Osakabe et al. 1999), while activity with ferulate was about 140 times lower (Table 3). Moreover, in mixed substrate assays, coniferylaldehyde was a potent inhibitor of ferulate hydroxylation. No activity in mixed assays was reported for feruloyl-CoA and coniferyl alcohol (Osakabe et al. 1999). In parallel, CYP84A1 from *Arabidopsis* was shown to catalyze the 5-hydroxylation of coniferyl aldehyde and also coniferyl alcohol with much higher efficiency compared to ferulate, with enzymatic properties rendering a physiological role of ferulate 5-hydroxylation by CYP84A1 unlikely (Humphreys et al. 1999, Table 3). Together, these results strongly suggest that the 5-hydroxylation occurs predominantly, if not exclusively, on the level of the aldehyde and/or alcohols and not, as previously assumed, on the level of the free acid. We thus prefer to use the abbreviation CA5H (coniferylaldehyde 5-hydroxylase), but will continue using published names (F5H, Cald5H, fah1) to avoid confusion with the cited literature.

Supporting these new data, microsomal fractions from sweetgum xylem extracts were then shown to hydroxylate coniferylaldehyde with much higher activity compared to ferulate (Osakabe et al. 1999). Furthermore, both Osakabe et al. (1999) and Humphreys et al. (1999) showed that caffeate *O*-methyl transferase (COMT), both in enzyme fractions from stems and when using the recombinant proteins, can efficiently convert 5-hydroxyconiferylaldehyde (and coniferylalcohol in the case of *Arabidopsis*) to sinapaldehyde, the precursor of sinapylalcohol and S-lignin. These surprising results raised the question how sinapic acid, which is the precursor of sinapate esters that are lacking in *fah1* mutants, is synthesized and why *fah1* mutants accumulate ferulic acid (Chapple et al. 1992). Analysis of the *Arabidopsis reduced epidermal fluorescence 1* mutant may answer this question as the *ref1* mutants has reduced sinapate and ferulate ester contents and reduced sinapaldehyde/coniferaldehyde dehydrogenase (SALDH/CALDH) activities (Nair et al. 2004). The corresponding wild type gene encodes a bifunctional SALDH/CALDH, and such activity was also found in other plant lineages (Nair et al. 2004). Together with results obtained regarding C3′H, this shows that the prevalent pathway to ferulic and sinapic acid proceeds from 4-coumarate via CoA-activation, shikimate-*trans*-esterification, 3-hydroxylation, reduction to the aldehyde, methoxylation, 5-hydroxylation, and dehydrogenation back to the free acid (Fig. 1). It needs further exploration to explain why this more energy consuming pathway compared to direct hydroxylations and methoxylations of the free acids has been favored by evolution. Also, residual amounts of ferulate and sinapate esters in the *ref1* mutant indicate the presence of an alternative pathway (Nair et al. 2004).

Table 3 Substrates analyzed and enzymatic properties of characterized CYP84s encoding coniferylaldehyde 5-hydroxylase (CA5H)

Enzyme	 ferulate			 feruloyl-CoA			 coniferyl aldehyde			 coniferyl alcohol			Reference
	K_M[a]	k_{cat}	k_{cat}/K_M	K_M	k_{cat}	k_{cat}/K_M	K_M	k_{cat}	k_{cat}/K_M	K_M	k_{cat}	k_{cat}/K_M	
CYP84A3 (*Ls*)	286	3.1	0.01	No activity in mixed assay			2.8	4.3	1.6	No activity in mixed assay			Osakabe et al. (1999)
	No activity in mixed assay[b]												
	K_M	V_{max}	V_{max}/K_M	K_M	V_{max}	V_{max}/K_M	K_M	V_{max}	V_{max}/K_M	K_M	V_{max}	V_{max}/K_M	
CYP84A1 (*At*)	1000	4.0	0.004	No activity			1.0	5.0	5.0	3.0	6.0	2.0	Humphreys et al. (1999)

Species abbreviations. *Liquidambar styraciflua* (sweetgum, *Ls*) and *Arabidopsis thaliana* (*At*)

[a] *Units*. K_M (μM); k_{cat} (min^{-1}); k_{cat}/K_M (min^{-1} μM^{-1}); V_{max} (pkat mg^{-1}); V_{max}/K_M (pkat mg^{-1} μM^{-1})

[b] For CYP84A3 some substrates were tested also in mixed substrate assays using equimolar concentrations of four potential substrates

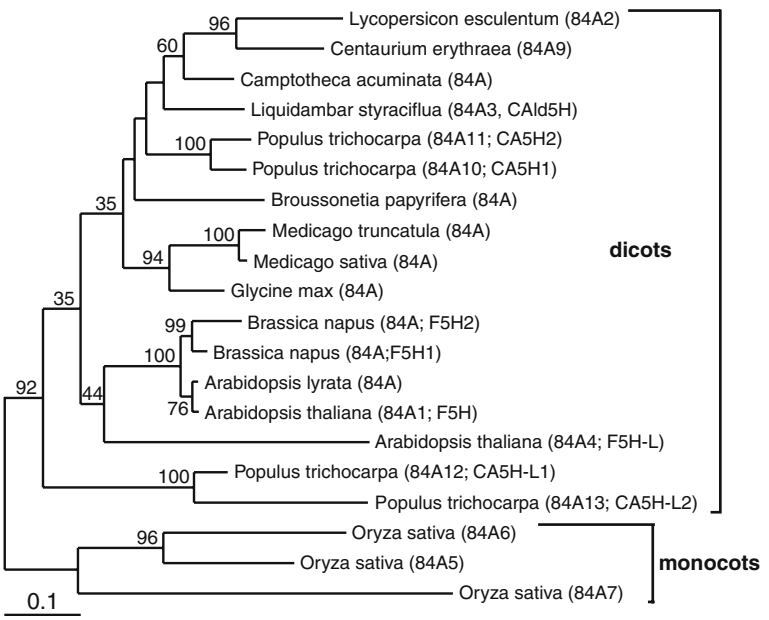


Fig. 4 Phylogenetic reconstruction of the CYP84A (CA5H) family. An alignment of full length protein sequences was generated using Dialign (Morgenstern 2004) and unequivocally alligned regions were used for a maximum likelihood analysis using Phylip (Felsenstein, 2005). Bootsrap values from 100 replicates found in more than 50 replicates are given at the nodes. When more divergent (CYP71) sequences were included, the three CYP84s from rice (*Oryza sativa*) were found at the base of the CYP84 clade and were thus used to root this tree

Phylogeny

An additional 18 *CYP84* full length sequences have been deposited into databases and phylogenetic reconstructions (Fig. 4) showed that the three *CYP84*s present in the rice (*Oryza sativa*) genome (International Rice Genome Project 2005) group at the base of the dicot sequences. Interestingly, the poplar genome (Joint Genomics Institute, *Populus trichocarpa* v. 1.0; http://genome.jgi-psf.org/Poptr1/Poptr1.home.html) contains four copies forming two distinct clades possibly suggesting divergent physiological functions (Fig. 4). All other putative CA5H sequences from dicots form a monophyletic cluster containing also the characterized CA5Hs from *Arabidopsis* and sweetgum. The *Arabidopsis* genome contains a second *CYP84* (Raes et al. 2003), which groups at the base of the Brassicacea cluster separated by a fairly large evolutionary distance (Fig. 4). None of the more divergent CYP84 members has been characterized to date, but it is obvious that CYP84A4, the *Arabidopsis* homolog (CA5H-L), cannot complement the *fah1* phenotype, either due to different enzymatic activities or due to exclusive expression patterns. Expression of *CA5H-L* under the control of the *CA5H* promoter might answer this question. It is interesting to note that no CYP84 gene has been identified in large scale EST projects of conifers. As gymnosperm lignin is usually devoid of S-subunits in their lignin, this is consistent with a function of CA5H specifically in S-lignin biosynthesis. Also, no CYP84 members were identified in the moss *Physcomitrella patens* together suggesting that CYP84 is specific to angiosperms coinciding with the appearance of S-lignin in this plant lineage.

Gene expression

Expression data for all *CYP84*s analyzed to date are consistent with a role in lignification with predominant expression in stems and roots observed for *F5H*s from *Arabidopsis* (Ruegger et al. 1999) and *Camptotheca acuminata* (Kim et al. 2006), in stems of *Brassica napus* (Nair et al. 2000), and in wood and bark of *Populus tremula* x *alba* (Sibout et al. 2002). Expression of *F5H* in *Arabidopsis* is limited to the lower parts of inflorescence stems, which are rich in interfascicular fibres that exclusively accumulate S-lignin (Meyer et al. 1998). The *C. acuminata F5H1* was also shown to be induced by wounding, a response that was enhanced by additional treatment with ethylene, methyl jasmonate, or hydrogen peroxide. In contrast, the wounding response was inhibited by salicylic acid (possibly acting as a jasmonate antagonist) and the NADPH-oxidase inhibitor diphenylene iodonium, but not by the ethylene-synthesis inhibitor aminoethoxyvinylglycine, suggesting that wound induced expression might be primarily controlled by jasmonate mediated signaling (Kim et al. 2006). However, no wound-induced expression of *F5H* from *Arabidopsis* was detectable (Ruegger et al. 1999), and despite the absence of CA5H activity in *fah1* mutants of *Arabidopsis*, no significant impact on lesion formation caused by *Botrytis cinerea* infection was observed (Kliebenstein et al. 2005). Distinct but overlapping expression patterns have been observed for three *CA5H* orthologs in canola (*Brassica napus*). All three isoforms are expressed predominantly in stems, but *F5H1* and *F5H2*, which are highly similar and are likely derived from orthologs in the ancestors of canola, namely *B. rapa* and *B. oleracea*, are also expressed to higher levels in leaves and, in particular *F5H2*, in seeds (Nair et al. 2000).

A detailed study compared the expression pattern of the *Arabidopsis F5H* gene in developing seedlings and siliques with that of other phenylpropanoid genes (Ruegger et al. 1999). These authors correlated *F5H* expression with sinapate ester biosynthesis, and showed overlapping, but temporally distinct, expression of *F5H* compared to other phenylpropanoid genes. Ruegger et al. (1999) also showed that *F5H* expression is not a limiting factor in sinapate ester biosynthesis, since over-expression of the gene does not lead to an accumulation of these compounds. Although not limiting, F5H is clearly necessary for the biosynthesis of sinapate esters (Chapple et al. 1992), which act as effective sunscreens against UV-irradiation. The *fah1* mutant, defective in *F5H*, is more sensitive to UV-B irradiation, and displayed leaf injury and enhanced oxydative damage of lipids and proteins at fluency rates that had no obvious effect on wild type plants. Rather than

flavonoids, sinapate esters appear as the major sunscreens in *Arabidopsis* (Landry et al. 1995).

Role in lignification

An impressive body of evidence exists, generated mainly by genetic and transgenic studies using the *Arabidopsis F5H* gene, that shows the central role of *F5H* in regulating flux into the syringyl fraction of lignin. Chapple et al. (1992) already reported the absence of S-lignin in the *sin1* (*fah1*) mutant. In wild-type *Arabidopsis*, syringyl units comprise about 20% of the lignin polymer, which are restricted to the sclerified parenchyma of the interfascicular region in inflorescence stems. In contrast, *fah1* mutants contain no detectable S-lignin and accumulate only G-lignin as determined by nitrobenzene oxidation and by the DFRC (Derivatization Followed by Reductive Cleavage) method (Meyer et al. 1998) and NMR (Marita et al. 1999). Ectopic expression of *F5H* under control of the *35S* promoter in the *fah1* background abolished the tissue specificity of lignin monomer deposition and resulted in an increase in S-lignin, although based on NMR analysis no major differences in lignin composition were found (Meyer et al. 1998; Marita et al. 1999). However, expression of *F5H* under control of the *C4H* promoter, which drives expression to all lignified tissues, resulted in a lignin that consisted almost entirely of syringyl subunits, which is mostly composed of β-aryl ether units. Such a simple, but extremely S-rich lignin has not been reported from any other plant. These data clearly demonstrate that *F5H* expression is responsible for quantitative and developmental regulation of lignin composition in *Arabidopsis* by limiting flux into sinapyl alcohol and subsequently to S-lignin. They also show that CYP84A1 is the sole CA5H involved in developmental lignin biosynthesis, and that the CYP84A4 homolog cannot complement this function.

Using the *C4H* promoter for over-expression of *F5H* from *Arabidopsis* in transgenic tobacco (*Nicotiana tabacum*) and poplar (*Populus tremula* × *alba*) leads to an increased syringyl proportion in secondary woody tissues, concomitant with a decrease in total Klason extractable lignin (Franke et al. 2000). Stems from one year old poplar trees had a ~65 mol% syringyl monomer content, while transgenic trees contained up to 93.5 mol% syringyl, on the basis of thioacidolysis. *F5H*-expression, however, did not alter total lignin content in older plants, but soluble lignin appeared to be increased. Transgene expression did not impact cell wall polysaccharide deposition and had no visible effect on wood morphology (Huntley et al. 2003). By comparing different kraft pulping conditions, these authors concluded that use of transgenic trees could increase pulp throughputs at mills by >60%, while concurrently decreasing chemicals and, consequently, the amount of deleterious byproducts released into the environment.

In parallel, it was shown that over-expression of the sweetgum *CAld5H* (*CYP84A3*) under control of a xylem specific *4CL* promoter in aspen (*Populus tremuloides*) also led to an increased S-proportion in lignin from developing xylem (Li et al. 2003). Again, total lignin content based on the Klason method was not reduced in these transgenic trees. Only when, in addition, 4CL activity was down-regulated by anti-sense suppression, which on itself reduces total lignin without altering the S/G ratio, a combination of both effects was observed, i.e., a reduced total lignin with an increased proportion of syringyl units. Furthermore, cell wall thickening appears to proceed faster in *CAld5H* over-expressing plants during xylem differentiation (Li et al. 2003). In reverse, over-expression of a poplar *CA5H* (*CYP84A10*) under control of a *35S* promoter in *Arabidopsis* wild type and *fah1* mutants resulted in complementation of the *fah1* phenotype, and an increase in syringyl lignin, in particular in roots of wild type plants (Sibout et al. 2002). Down-regulation of *CA5H* in alfalfa (*Medicago sativa*) by anti-sense expression of the *F5H-K10* gene under control of the vascular specific *PAL*-promoter led to a twofold decreased S-lignin content (Reddy et al. 2005), and anti-sense (and sense) expression of the *F5H1* gene in canola (*Brassica napus*), both under control of the seed specific *napin*- or the *35S*-promoter, resulted in reduction of the sinapate ester sinapine in seeds (Nair et al. 2000). Finally, down-regultion of *F5H* in tomato (*Lycopersicon esculentum*) results in increased chlorogenic acid content in pericarp and an increase in relative flavonoid

contents in skin tissue of the fruits (Long et al. 2006), also indicating a cross-talk between monolignol and flavonoid biosynthesis.

In summary, both the genetic and biochemical studies clearly show that members of the CYP84 clade are required for sinapate-ester biosynthesis, are limiting factors regulating flux into syringyl lignin, and act predominantly, if not exclusively, on the level of the aldehyde (and alcohol) of 3-methoxylated phenylpropanoids. Given this specific function, *CA5H* genes thus are suitable for engineering both lignin composition and sinapate esters contents in commercially important species.

Biosynthesis of lignan derivatives (podophyllotoxin and 6-methoxypodophyllotoxin)

Lignans, widespread phenolic metabolites in the plant kingdom, are derived by C8–C8′ oxidative dimerization of phenylethanoid compounds such as caffeoyl-, coniferyl- or sinapoyl-alcohol and thereby share precursors with lignin biosynthesis. The primary lignan is subsequently modified by ring cleavage and formation reactions, and a diverse range of hydroxylations and further substitutions give rise to a family of immense structural multiplicity.

These compounds have been of major scientific interest since the early days of medical research as they were shown to possess a great variety of biological and pharmacological activities. In the last 15 years more than 120 lignans were reported to have anti-inflammatory, antimicrobial, immunosuppressive, anticancer, and antioxidative activity (reviewed by Saleem et al. 2005) explaining the newly sparked interest.

A group that has proven particularly successful in cancer chemotherapy were semi-synthetic derivatives of podophyllotoxin (PTOX). E.g., etoposide, teniposide, and etopophos inhibit the activity of topoisomerase II thereby impairing cell division. PTOX itself inhibits microtubule formation and has severe toxic side-effects. It therefore only serves as starting material for the semi-synthesis of anticancer drugs (Canel et al. 2000). Podophyllotoxin is being isolated from rhizomes of *Podophyllum* species (Podophyllaceae) collected from wild habitats. For that reason, the evaluation of alternative biotechnological resources and the investigation of the biosynthesis of PTOX and derivatives have been extended to *Linum* species and cell cultures. The latter build up PTOX and predominantly 6-methoxypodophyllotoxin (MPTOX) as glucosides, among other lignans (Molog et al. 2001; Kuhlmann et al. 2002).

The biosynthesis of both compounds bifurcates at deoxypodophyllotoxin, which is either hydroxylated at the 7th position of the C ring to PTOX, or alternatively in a series of reactions beginning with hydroxylation of the aromatic B ring in 6th position yielding β-peltatin. Subsequent *O*-methyl transfer, and finally a C ring hydroxylation analogously to PTOX formation result in MPTOX (Fig. 5). Molog et al. (2001) reported the initial hydroxylation in MPTOX biosynthesis in *Linium flavum* to be catalysed by the deoxypodophyllotoxin 6-hydroxylase (DOP6H). The reaction required NADPH and molecular oxygen, and the enzyme was localized in the microsomal fraction. Further data showing a strong inhibition of DOP6H by cytochrome *c* and a partially reversible inhibition by carbon monoxide supported the assumption that DOP6H represents a P450 enzyme. Activity for the second hydroxylation step, at the aliphatic C-ring, could not be found in these microsomal fractions.

Currently, three semi-synthetic *O*-glycosidic derivatives of PTOX are widely used as antitumor agents (etoposide, teniposide, and etopophos). One of the crucial conversion steps from the cytotoxic PTOX to therapeutic analogues is stereoselective β-glucosylation of the C-7 hydroxy function (Canel et al. 2000). The B-6-methoxy ring substitution of MPTOX might therefore sterically obstruct reactivity or destabilize the C-7 stereocenter and result in racemization, sufficiently impairing efficiency of the semi-synthesis with MPTOX as precursor to make the process unprofitable. On the other side, DOP6H activity is missing in *L. album*, which mainly accumulates PTOX. Manipulation of the MPTOX pathway by blocking the crucial first step of 6-hydroxylation might therefore redirect the metabolite flux towards PTOX, and thus help increasing the PTOX yield in *L. flavium* at cost of MPTOX. Cloning and molecular characterization of the

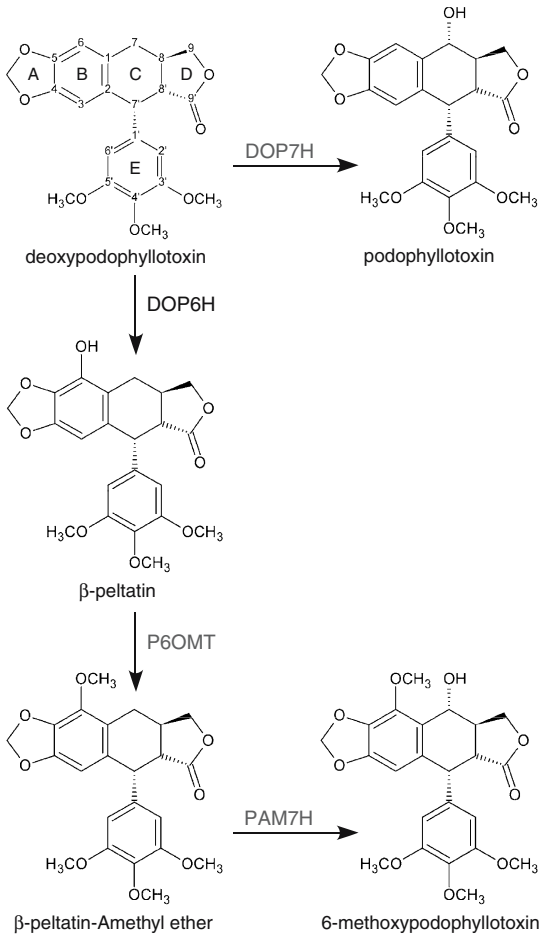


Fig. 5 Proposed biochemical pathway leading to podophyllotoxin and 6-methoxypodophyllotoxin. Pathway adapted from Molog et al. (2001). DOP7H: deoxypodophyllotoxin 7-hydroxylase; DOP6H: deoxypodophyllotoxin 6-hydroxylase; P6OMT: *β*-peltatin 6-*O*-methyltransferase; PAM7H: *β*-peltatin-A methylether 7-hydroxylase

L. flavium DOP6H P450 gene would represent the next necessary steps for targeted control of the in vivo enzyme activity on either gene, transcript or post-translational level.

Biosynthesis of hydroxyphenylethanol derivatives (salidroside and cornoside)

Phenylethanoids are classified according to their oxidation pattern of the aromatic ring. Beside lignan derivatives (see above) the second group represents 4-hydroxy phenylethanoid derivatives such as cornoside, which is presumed to be

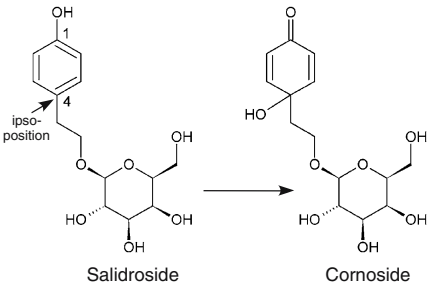


Fig. 6 Reaction catalyzed by salidroside oxydase

synthesized from 4-hydroxyphenylethanol via salidroside (Eigtved et al. 1976). Subsequently oxidation leads to the benzoquinol derivative (Fig. 1).

Salidroside oxidase

The term salidroside is derived from *Salix*, the genus name for willows. Salidroside has among other phenylethane derivatives been shown to be pharmacologically active as an antioxidant and adaptogen (Panossian and Wagner 2005). The biosynthesis occurs likely via tyrosol (Fig. 1) as shown in chemical feeding studies using cell culture (Wu et al. 2003). Oxidation of salidroside yields cornoside (Fig. 6), a compound with a limited distribution in plants found mainly in *Cornus* species (Jensen et al. 1975). Cornoside is currently used as chemosystematic and taxonomic marker, and its distribution seems mutually exclusive with iridoids, cyclic terpenoid derivatives (Jensen et al. 2005; Taskova et al. 2005).

Cornoside biosynthesis was investigated in four oleaceous plants, namely white forsythia (*Abeliophyllum distichum*), *Forsythia suspensa, F. viridissim*a, and *F. koreana*. Among these, *A. distichum* was found to contain the highest amount of cornoside, representing the major secondary metabolite (Yamamoto et al. 1998). Yamamoto et al. (2003) further investigated the oxidative conversion of salidroside to cornoside in *A. distichum* cell cultures. Microsomal fractions were shown to convert salidroside to cornoside, when incubated with NADPH. The enzyme is specific for its substrate salidroside and requires molecular oxygen for cornoside formation. Carbon monoxide strongly and reversibly inhibited

the activity in the dark, as did cytochrome c and several P450 inhibitors, suggesting that the salidroside oxidation is catalyzed by a P450 enzyme. Even though the quinol formation was mechanistically expected because of the 4-substitution with a residue that is unlikely to eliminate (Ohe et al. 1997), the *A. distichum* P450 represents the first example reported for a cytochrome P450 monooxygenase catalyzing the formation of a benzoquinol in plants. Cloning of the P450 gene and comparison with existing closely related P450s in conjunction with phylogenetic analyses might corroborate the valuable role of cornoside as chemotaxonomic marker and complement the systematic investigations on the molecular level in the future.

Biosynthesis of salicylic acid and benzoate derivatives

Salicylic acid (2-hydroxybenzoic acid) is one of the key signal molecules in plant defense. Systemic acquired resistance and localized cell death, crucial defense responses of plants to pathogens require salicylic acid. Several routes for the biosynthesis of benzoic acid derivatives were characterized in plants and may be specific for species, tissues or responses (Ogawa et al. 2005) or they may even operate in parallel, temporally separated pathways (Boatright et al. 2004).

''Benzoic acid 2-hydroxylase'' (BA2H)

Upon pathogen or elicitor treatment, in tobacco and cucumber salicylic acid biosynthesis was proposed to proceed exclusively, and in potato predominantly, from *trans*-cinnamic acid by oxidative decarboxylation to benzoic acid and subsequent 2-hydroxylation of benzoic acid to salicylic acid (Yalpani et al. 1993; Meuwly et al. 1995; Coquoz et al. 1998). The final step is catalyzed by benzoic acid 2-hydroxylase (BA2H), an enzyme that was found constitutively expressed in tobacco and highly induced, in coordination with the phenylpropanoid pathway, after inoculation of tobacco leaves with tobacco mosaic virus (TMV). This activity was also induced upon application of benzoic acid (Leon et al. 1993) or ozone treatment (Pasqualini et al. 2002). It was shown that salicylic acid accumulation in TMV inoculated tobacco was inhibited by CO, and that tetcyclacis, a specific P450 inhibitor, caused a strong increase of free benzoic acid accumulation, with concomitant reduction of free salicylic acid in TMV inoculated plants compared to controls. The 2-hydroxylase activity was detected almost exclusively in the soluble fraction, whereas 4-hydroxylation of benzoate still was catalyzed by the microsomal protein fraction (Leon et al. 1995). The authors report a partial purification of a 160 kDa protein corresponding to BA2H activity. Immunoprecipitation of L-[35S]methionine labeled proteins from mock- and TMV-inoculated tobacco leaves with anti-SU2 raised against a presumably highly divergent, soluble *Streptomyces griseolus* P450 resulted accordingly in specific high molecular weight bands.

The report of a soluble, catalytically self-sufficient tobacco P450 of high molecular weight represents a unique case in the eucaryotic world, where, with the exception of a small number of fungal P450s, all proteins characterized so far represent 50–60 kDa membrane bound proteins requiring autonomous P450 reductases (CPR). The increased molecular weight and the enzymatic properties of the tobacco BA2H might therefore be an indication of a fusion protein with its own NADPH-reductase domain. Even though numerous examples of bifunctional P450:NADPH-P450 reductases exist in bacteria and fungi, no plant sequences could be found with P450 and CPR domain located on the same peptide in global or partial genome sequencing data. Without exception, all plant sequences identified so far based on cloned sequences of bona fide P450 or CPR, on similarity to known enzymes, or on the presence of the characteristic motives are exclusively singular and shorter compared to the bi-partite self-sufficient fungal and bacterial counterparts.

The tobacco BA2H is, according to the authors' findings, likely playing a crucial role in salicylic acid biosynthesis and is related to plant defense. It was shown in *Arabidopsis* that production of salicylic acid precursors is a function of PAL in the resistance to *Peronospora parasitica* (Mauch-Mani and Slusarenko 1996), therefore

following a biosynthetic route comparable to tobacco. However, the fact, that no candidate genes analogous to the described tobacco *BA2H* were identified yet, despite the immense variety of pathogen-interaction and salicylate signal transduction pathway mutant screens, as well as the lack of reports reproducing BA2H in other organisms could at least point out, that the BA2H reported by Leon et al. (1995) represents a tobacco specific rarity. It must however be kept in mind that recently a cinnamate independent pathway was identified that forms salicylic acid via chorismate to isochorismate by isochorismate synthase (ICS) and isochorismate pyruvate lyase (IPL) (Wildermuth et al. 2001). This pathway appears to be the predominant pathway to form salicylic acid in *A. thaliana*. This may complicate the isolation of mutant lines affected in an alternative pathway because of the potential functional redundancy in some organisms.

Other points also raise doubts about the P450 nature of the BA2H protein. Antibodies raised against the eucaryotic avocado P450 (ARP1) could not interact with BA2H, while antibodies raised against the bacterial SU2 with no significant homology to plant P450 sequences was efficient to immunodeplete the tobacco BA2H. This is in particular surprising considering that the SU2 antibodies were shown not to interact with the bacterial P450 SU1 protein from the same P450 family, catalyzing similar reactions in *Streptomyces griseolus* (Omer et al. 1990). In addition, no other P450 was ever reported to be able to turnover with an electron donor reduced by methyl viologen. At the present stage, the tobacco BA2H represents the only soluble, high molecular weight plant P450 reported, which at the same time does not require the autonomous action of a CPR. Until characterized on the molecular level, it thus remains an open question if BA2H is truly a bona fide P450-like enzyme. Protein sequencing of the tobacco BA2H or isolation of the corresponding gene would bring all future speculations to an end.

''Benzoic acid 4-hydroxylase'' (BA4H)

Leon et al. (1995) reported in the same manuscript a strong benzoate 4-hydroxylase activity restricted to the microsomal fraction. Analogously to 2-hydroxy-benzoate, the biosynthetic route to 4-hydroxy-benzoate has been shown to proceed via different pathways in different species. Schnitzler et al. (1992) reported the non-oxidative side chain degradation of 4-coumarate via 4-hydroxybenzaldehyde, and its conversion to 4-hydroxy-benzoate in elicited carrot (*Daucus carota*) cell extracts by a NAD-dependent dehydrogenase. Löscher and Heide (1994) described the biosynthesis of 4-hydroxybenzoate from 4-coumarate and 4-coumaroyl-CoA in cell-free extracts of *Lithospermum erythrorhizon* cell cultures, in analogy to the NAD-dependent fatty acid *beta*-oxidation. Funk and Brodelius (1994) proposed a complex process involving 4-methylation, glucosylation, side chain shortening, and 4-demethylation in *Vanilla planifolia* cell culture. Finally, recent evidence presented by Wildermuth et al. (2001) supports an alternative route straight from the shikimate pathway precursors to a limited number of benzoate derivatives, in a similar process as found in bacteria. None of the cinnamoyl dependent routes involves hydroxylation steps with free benzoic acid, they instead take advantage of 4-coumarate generated by *C4H* (see above). Contribution of a second, independent P450 in the generation of 4-hydroxy-benzoate seems unlikely in plants given the immense metabolic flux through the phenylpropanoid pathway, which appears to provide more than adequate levels of 4-hydroxylated precursors. The situation is different in fungi, where phenylpropanoid metabolism plays an entirely different role. The subfamily CYP53B, represented by the fungal microsomal BA4H from the ascomycete *Aspergillus niger* (van Gorcom et al. 1990), from the basidiomycete *Rhodotorula minuta* (Fujii et al. 1997) and the lignin-degrading basidiomycete *Phanerochaete chrysosporium* (Matsuzaki and Wariishi 2005), clearly shows P450 dependent conversion of benzoate to 4-hydroxybenzoate. Comparison of these sequences against plant genomes resulted in P450 homologues with divergent functions in phenolic metabolism, i.e. flavonoid 3′-hydroxylation and metabolism of phenylurea, implicating either the lack of a plant P450 BA4H or convergent evolution.

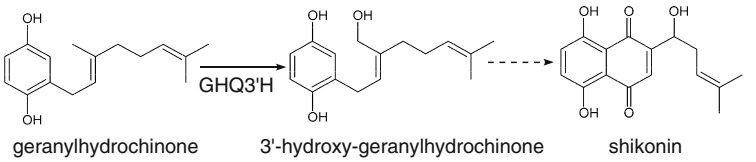


Fig. 7 Reaction catalyzed by geranylhydroquinone 3′-hydroxylase (GHQ3′H). Shown is the reaction leading to 3′-hydroxy-geranylhydroquinone, which is an intermediate in the biosynthesis of shikonin. Multiple enzymatic steps are indicated by a dashed arrow

Other benzoate hydroxylases

There are a few additional steps with yet uncharacterized or speculated reactions potentially involving plant cytochrome P450s. A novel 2-hydroxybenzoate 5-hydroxylase was identified and characterized from *Catharanthus roseus* (Shimoda et al. 2002). Even though the 2-hydroxy-benzoate 5-hydroxylase was not particularly tested for characteristic P450 attributes, it showed sequence similarity to certain plant flavonoid 3′-hydroxylases and used NADPH as a coenzyme (Shimoda et al. 2004). The enzyme activity was inhibited by the two-valent copper and mercury cations, and seemingly enhanced by iron. Nevertheless, the enzyme was assayed for inactivation by diethylpyrocarbonate (DEPC), which can selectively modify unprotonated amino acids such as the conserved cysteine in the P450 heme binding domain, but the protein remained active even when it was treated with 50 mM DEPC (Shimoda et al. 2004).

Quinoid compounds (shikonin biosynthesis)

Ubiquinone or Coenzyme Q is a pivotal, lipid-soluble coenzyme that transports electrons in the respiratory chains of both prokaryotes and eukaryotes. Ubiquinone biosynthesis in eukaryotes, as initially deduced from reactions in yeast proceeds from 4-hydroxybenzoic acid via 3-polyprenyl-4-hydroxybenzoic acid (Momose and Rudney 1972). Downstream in the pathway several hydroxylation and other oxidation reactions occur, which have the potential to be catalyzed by cytochrome P450s. However, no enzymatic activities were characterized and/or the reactions are catalyzed by other oxygenases in fungi or bacteria. However, numerous other quinoid compounds exist in plants, some of which, e.g., shikonin, having medical importance. Shikonin is a red naphthazarin pigment derived from the roots of *Lithospermum erythorhizon*. Investigations of the metabolism of geranylhydroquinone in cell-free extracts from *L. erythrorhizon* cells, in order to identify the reactions preceding the final cyclization step in shikonin biosynthesis, led Yamamoto et al. (2000) to the discovery of geranylhydroquinone 3′-hydroxylase (GHQ3′H), a cytochrome P450 monooxygenase catalyzing the conversion of GHQ to its hydroxylated derivative 3′-hydroxy-geranylhydroquinone, which is an intermediate in the biosynthesis of shikonin (Fig. 7). The microsomally localized enzyme required molecular oxygen and NADPH, was reversibly inhibited by carbon monoxide in the dark and cytochrome *c* as well as other P450 inhibitors. Yet, the final cyclization reaction, as well as the gene coding for the *Lithospermum* GHQ3′H remains to be characterized.

Xanthone biosynthesis

Xanthones are yellow pigments of phenolic origin usually occurring in a few families of higher plants, some fungi and lichens. Several hundred derivatives have been identified so far (reviewed by Peres et al. 2000). In plants they may be synthesized as protective agents, as shown by Koiwai et al. (1974), in response to ozone injury. Xanthones are of great interest as potential pharmaceuticals for a broad variety of medical treatments. For example Garcinone E, has been shown to be a potent cytotoxic against hepatocellular carcinoma cell lines (Ho et al. 2002), and xanthones from *Calophyllum caledonicum, Andrographis paniculata, Garcinia cowa* and *G. mangostana* possess antimalarial, antimicrobial and antifungal activity (Hay et al. 2004; Dua

et al. 2004; Likhitwitayawuid et al. 1998; Gopalakrishnan et al. 1997). Other xanthones (mangostin and analogues) inhibit stages in the replication cycle of human immunodeficiency virus (HIV) by protease inhibition (Vlietinck et al. 1998).

The entry molecule 3-hydroxybenzoate for benzophenone biosynthesis (Fig. 1), the immediate precursor of xanthone has been shown to be derived from two independent pathways, either from the phenylpropanoid pathway or from an early step of the shikimate pathway (Abd El-Mawla et al. 2001; Wang et al. 2003). The 3-hydroxybenzoate is the preferred substrate of the *Centaurium erythraea* benzophenone synthase (BS), which catalyzes in analogy to chalcone synthase the sequential condensation of three molecules of malonyl-CoA with 3-hydroxybenzoyl-CoA to form 2,3′,4,6-tetrahydroxybenzophenone (THBP; Fig. 8; Beerhues 1996).

Benzophenone 3′-hydroxylase (B3′H)

Alternatively to this route, benzoic acid itself has been shown to be a precursor of the central key metabolite THBP. As demonstrated in *Hypericum androsaemum*, benzoic acid is converted in a series of reactions involving first a benzoate:CoA-ligase (which also has the capability to accept 4-hydroxybenzoate and 3-hydroxybenzoate), then a benzophenone synthase with high specificity to benzoyl-CoA, and ultimately a benzophenone 3′-hydroxylase (B3′H) to form THBP (Fig. 8 and Schmidt and Beerhues 1997). The key step, conversion of 2,4,6-trihydroxybenzophenone to THBP was shown by Schmidt and Beerhues (1997) to be catalyzed by B3′H, an enzyme of the microsomal fraction isolated from cell-cultures of *H. androsaemum*, requiring NADPH and depending on molecular oxygen. Furthermore, the enzyme was strongly inhibited by known P450 inhibitors and reversibly by a $CO:O_2$ gas mixture in the dark, demonstrating that the enzymatic B3′H activity detected was due to a P450 monooxygenase. The activity of the B3′H was highest with 2,4,6-trihydroxybenzophenone, and dropped significantly whith dihydroxylated or monohydroxylated benzophenone. Engineering of the xanthone pathway requires all steps, especially the

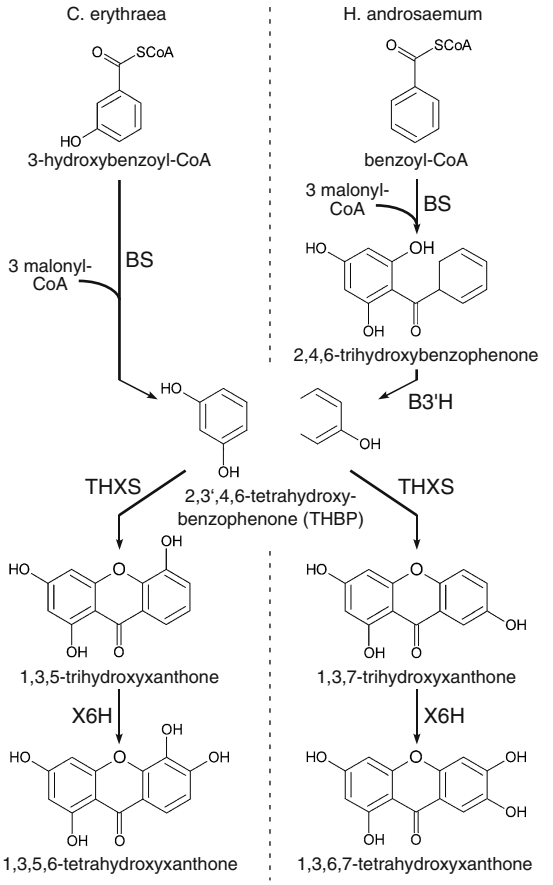


Fig. 8 Xanthone biosynthesis in *Centaurium erythraea* and *Hypericum androsaemum*. Shown are the preferred substrates in each species of benzophenone synthase (BS), benzophenone 3′-hydroxylase (B3′H), trihydroxyxanthone synthase (THXS), and xanthone 6-hydroxylase (X6H)

potentially rate-limiting oxidoreductase steps coupled to electron transfer chains to be mechanistically resolved. However, the B3′H P450 gene still remains to be cloned and biochemically characterized in detail.

Trihydroxyxanthone synthase (THXS)

The resulting THBP derived via either pathways is subsequently intramolecularly and regioselectively coupled to form 1,3,5-trihydroxyxanthone, or to the isomeric form 1,3,7-trihydroxyxanthone (Fig. 8) by a strictly NADPH dependent enzyme of cultured cells of *C. erythraea* or *H. androsaemum*, respectively (Peters et al. 1997). These reactions occur likely by an oxidative phenol

coupling reaction mechanism, and are catalyzed by trihydroxyxanthone synthases (THXS) localized in the microsomal fractions. The enzymatic activity depends on molecular oxygen and displays blue-light-reversible carbon monoxide inhibition and sensitivity to P450 inhibitors, but not to peroxidase inhibitors. Finally, presence of P450 activity in the microsomal fractions from both *C. erythraea* and *H. androsaemum* was confirmed by the typical carbon monoxide difference spectra of microsomes. The THXS were for that reason proposed to be cytochromes P450 (Peters et al. 1997). Molecular characterization of the THXS genes will help determining the extent of the gene families in both species. In addition, cloning of the THXS genes and site-directed mutagenesis might allow interchanging specificity, and thereby help to elucidate the mechanism for the regioselective coupling.

Xanthone 6-hydroxylase (X6H)

In a subsequent step following the xanthone ring formation, Schmidt et al. (2000) reported the activity of a xanthone 6-hydroxylase, responsible for the generation of the major constituents, which are a mixture of 1,3,6,(5/7)-tetrahydroxyxanthone derivatives in *H. androsaemum*, and higher substituted 3,5,6,7,8-pentamethoxy-1-*O*-primeverosylxanthone in *C. erythraea*. The enzyme activity was found in the microsomal fraction, and was shown to be dependent on NADPH. The hydroxylation reactions required molecular oxygen, and were blocked by P450 inhibitors and light-reversibly affected by CO, supporting the authors' conclusion that X6Hs are P450 enzymes. Interestingly, and in accordance with the results of the B3′H activities and with the accumulation of xanthone products, the *H. androsaemum* X6H accepted both 1,3,5-trihydroxyxanthone and 1,3,7-trihydroxyxanthone. With the former having 65% of the activity observed with the latter. In contrast, only 1,3,5-trihydroxyxanthone was accepted by the *C. erythraea* X6H.

Finally, the occurrence of the higher substituted xanthone in *C. erythraea* might indicate the presence of further P450s or alternative oxidoreductases. To date, none of the genes coding for the oxidative steps in *H. androsaemum* and *C. erythraea* has been cloned. Different hydroxylation patterns were found in members of the Liliaceae family (Gupta and Lewis 1971; Fujita and Inoue 1981; Bennett et al. 1990), where most likely similar enzymes are responsible for the analogous biosynthetic steps. In depth biochemical characterization of the xanthone biosynthesis pathway in the species, in combination with sequence information of the P450s involved are crucial prerequisites for successful metabolic engineering of the pharmaceutically important biosynthetic pathway(s) leading to the great variety of xanthones.

Conclusions

The advances achieved in the recent years have led to characterization of the three major cytochromes P450 which catalyze hydroxylations of the phenolic ring for the synthesis of lignin precursors and other compounds derived from the main phenylpropanoid pathway. This characterization revealed an unexpected complexity in the main pathway, and it also showed that the P450s of the phenylpropanoid pathway are excelent targets for metabolic engineering of crop plants. However, these novel results also indicated that the phenolic metabolism with its different ramifications is still very far from being fully understood. The major points remaining to be resolved include the specific functions *in planta* of the duplications of the *CYP73*, *CYP98* and *CYP84* genes in various plant species, the potential catalytic plasticity of some of the enzymes, and the alternative routes which appear to be activated upon blockage of the main pathway. Genetic studies also showed that our understanding of the roles and impact of phenylpropanoids for plant development and adaptation is still in its infancy. Although a few of the P450s catalyzing downstream reactions in the various branch pathways have been revealed at the enzymatic or molecular level, a plethora of them still need to be characterized, some in potentially critical and ubiquitous branch pathways, such as that of benzoic acids or ubiquinone biosynthesis.

Acknowledgements We would like to thank the following agencies for financial support of the authors. RM-R is supported by a doctoral student stipend from the Région Alsace (04/908/19/277) and JE by a Marie Curie grant from the European Union (IRG36537).

References

Abd El-Mawla AMA, Schmidt W, Beerhues L (2001) Cinnamic acid is a precursor of benzoic acids in cell cultures of *Hypericum androsaemum* L. but not in cell cultures of *Centaurium erythraea* RAFN. Planta 212:288–293

Abdulrazzak N, Pollet B, Ehlting J, Larsen K, Asnaghi C, Ronseau S, Proux C, Erhardt M, Seltzer V, Renou JP, Ullmann P, Pauly M, Lapierre C, Werck-Reichhart D (2006) A coumaroyl-ester-3-hydroxylase insertion mutant reveals the existence of nonredundant *meta*-hydroxylation pathways and essential roles for phenolic precursors in cell expansion and plant growth. Plant Physiol 140:30–48

Achnine L, Blancaflor EB, Rasmussen S, Dixon RA (2004) Colocalization of L-phenylalanine ammonia-lyase and cinnamate 4-hydroxylase for metabolic channeling in phenylpropanoid biosynthesis. Plant Cell 16:3098–3109

Anterola AM, Jeon JH, Davin LB, Lewis NG (2002) Transcriptional control of monolignol biosynthesis in *Pinus taeda*. (Factors affecting monolignol ratios and carbon allocations in phenylpropanoid metabolism). J Biol Chem 277:18272–18280

Anterola AM, Lewis NG (2002) Trends in lignin modification: a comprehensive analysis of the effects of genetic manipulations/mutations on lignification and vascular integrity. Phytochemistry 61:221–294

Ayabe S-I, Akashi T (2006) Cytochrome P450 in flavonoid metabolism. Phytochem Rev, this issue

Bartlett DJ, Poulton JE, Butt VS (1972) Hydroxylation of *p*-coumaric acid by illuminated chloroplasts from spinach beet leaves. FEBS Lett 23:265–267

Batard Y, Schalk M, Pierrel MA, Zimmerlin A, Durst F, Werck-Reichhart D (1997) Regulation of the cinnamate 4-hydroxylase (CYP73A1) in Jerusalem artichoke tubers in response to wounding and chemical treatments. Plant Physiol 113:951–959

Batard Y, Hehn A, Nedelkina S, Schalk M, Pallett K, Schaller H, Werck-Reichhart D (2000) Increasing expression of P450 and P450-reductase proteins from monocots in heterologous systems. Arch Biochem Biophys 379:161–169

Batz O, Logemann E, Reinold S, Hahlbrock K (1998) Extensive reprogramming of primary and secondary metabolism by fungal elicitor or infection in parsley cells. Biol Chem 379:1127–1135

Baucher M, Halpin C, Petit-Conil M, Boerjan W (2003) Lignin: genetic engineering and impact on pulping. Crit Rev Biochem Mol Biol 38:305–350

Beerhues L (1996) Benzophenone synthase from cultured cells of *Centaurium erythraea*. FEBS Lett 383:264–266

Bell-Lelong DA, Cusumano JC, Meyer K, Chapple C (1997) Cinnamate-4-hydroxylase expression in Arabidopsis (regulation in response to development and the environment). Plant Physiol 113:729–738

Bennett GJ, Lee H-H, Das NP (1990) Biosynthesis of mangostin. Part 1. The origin of the xanthone skeleton. J Chem Soc Perkin Trans 1:2671–2676

Bernards M, Lewis N (1998) The macromolecular aromatic domain in suberized tissue: a changing paradigm. Phytochemistry 47:915–933

Betz C, McCollum TG, Mayer RT (2001) Differential expression of two cinnamate 4-hydroxylase genes in 'Valencia' orange (*Citrus sinensis* Osbeck). Plant Mol Biol 46:741–748

Blee K, Choi JW, Connell AP, Jupe SC, Schuch W, Lewis NG, Bolwell GP (2001) Antisense and sense expression of cDNA coding for CYP73A15, a class II cinnamate 4-hydroxylase, leads to a delayed and reduced production of lignin in tobacco. Phytochemistry 57:1159–1166

Blount JW, Korth KL, Masoud SA, Rasmussen S, Lamb C, Dixon RA (2000) Altering expression of cinnamic acid 4-hydroxylase in transgenic plants provides evidence for a feedback loop at the entry point into the phenylpropanoid pathway. Plant Physiol 122:107–116

Blount JW, Masoud SA, Sumner L, Huhman D, Dixon RA (2002) Over-expression of cinnamate 4-hydroxylase leads to increased accumulation of acetosyringone in elicited tobacco cell-suspension cultures. Planta 214:902–910

Boatright J, Negre F, Chen X, Kish CM, Wood B, Peel G, Orlova I, Gang D, Rhodes D, Dudareva N (2004) Understanding in vivo benzenoid metabolism in petunia petal tissue. Plant Physiol 135:1993–2011

Boerjan W, Ralph J, Baucher M (2003) Lignin biosynthesis. Annu Rev Plant Biol 54:519–546

Boniwell J, Butt V (1986) Flavin nucleotide-dependent 3-hydroxylation of 4-hydroxyphenylpropanoid carboxylic acids by particulate preparations from potato tubers. Z Naturforsch 41:56–60

Bourgaud F, Hehn A, Larbat R, Doerper S, Gontier E, Kellner S, Matern U (2006) Biosynthesis of coumarins in plants: a major pathway still to be unravelled for cytochrome P450 enzymes. Phytochem Rev, this issue

Canel C, Moraes RM, Dayan FE, Ferreira D (2000) Podophyllotoxin. Phytochemistry 54:115–120

Chapple CCS, Vogt T, Ellis BE, Somerville CR (1992) An Arabidopsis mutant defective in the general phenylpropanoid pathway. Plant Cell 4:1413–1424

Coquoz JL, Buchala A, Metraux JP (1998) The biosynthesis of salicylic acid in potato plants. Plant Physiol 117:1095–1101

Costa MA, Bedgar DL, Moinuddin SGA, Kim KW, Cardenas CL, Cochrane FC, Shockey JM, Helms GL, Amakura Y, Takahashi H, Milhollan JK, Davin LB, Browse J, Lewis NG (2005) Characterization in vitro and in vivo of the putative multigene 4-coumarate:CoA ligase network in Arabidopsis: syringyl lignin and sinapate/sinapyl alcohol derivative formation. Phytochemistry 66:2072–2091

Dixon RA (2001) Natural products and plant disease resistance. Nature 411:843–847

Dua VK, Ojha VP, Roy R, Joshi BC, Valecha N, Devi CU, Bhatnagar MC, Sharma VP, Subbarao SK (2004) Anti-malarial activity of some xanthones isolated from the roots of *Andrographis paniculata*. J Ethnopharmacol 95:247–251

Ehlting J, Mattheus N, Aeschliman DS, Li E, Hamberger B, Cullis IF, Zhuang J, Kaneda M, Mansfield SD, Samuels L, Ritland K, Ellis BE, Bohlmann J, Douglas CJ (2005) Global transcript profiling of primary stems from Arabidopsis thaliana identifies candidate genes for missing links in lignin biosynthesis and transcriptional regulators of fiber differentiation. Plant J 42:618–640

Eigtved P, Jensen OS, Kjaer A, Wieczorkowska E (1976) Biosynthesis of a quinol glucoside in cornus. Acta Chem Scand B:182–184

Facchini PJ, Huber-Allanach KL, Tari LW (2000) Plant aromatic amino acid decarboxylases: evolution, biochemistry, regulation, and metabolic engineering applications. Phytochemistry 54:121–138

Fahrendorf T, Dixon RA (1993) Stress responses in alfalfa (*Medicago sativa* L.) XVIII: Molecular cloning and expression of the elicitor-inducible cinnamic acid 4-hydroxylase cytochrome P450. Arch Biochem Biophys 305:509–515

Fitzpatrick PF (2003) Mechanism of aromatic amino acid hydroxylation. Biochemistry 42:14083–14091

Frank MR, Deyneka JM, Schuler MA (1996) Cloning of wound-induced cytochrome P450 monooxygenases expressed in pea. Plant Physiol 110:1035–1046

Franke R, McMichael CM, Meyer K, Shirley AM, Cusumano JC, Chapple C (2000) Modified lignin in tobacco and poplar plants over-expressing the Arabidopsis gene encoding ferulate 5-hydroxylase. Plant J 22:223–234

Franke R, Hemm MR, Denault JW, Ruegger MO, Humphreys JM, Chapple C (2002a) Changes in secondary metabolism and deposition of an unusual lignin in the ref8 mutant of Arabidopsis. Plant J 30:47–59

Franke R, Humphreys JM, Hemm MR, Denault JW, Ruegger MO, Cusumano JC, Chapple C (2002b) The Arabidopsis REF8 gene encodes the 3-hydroxylase of phenylpropanoid metabolism. Plant J 30:33–45

Fujii T, Nakamura K, Shibuya K, Tanase S, Gotoh O, Ogawa T, Fukuda H (1997) Structural characterization of the gene and corresponding cDNA for the cytochrome P450rm from *Rhodotorula minuta* which catalyzes formation of isobutene and 4-hydroxylation of benzoate. Mol Gen Genet 256:115–120

Fujita M, Inoue T (1981) Further studies on the biosynthesis of mangiferin in anemarrhena asphodeloides: hydroxylation of the shikimate-derived ring. Phytochemistry 20:2183–2185

Funk C, Brodelius P (1994) *Vanilla Planifolia* Andrews: in vitro biosynthesis of vanillin and other phenylpropanoid derivatives. In: Bajaj YPS (ed) Medicinal and aromatic plants VI. Biotechnology in agriculture and forestry, vol 26. Springer-Verlag, Berlin, Heidelberg, New York, pp. 377–402

Gang DR, Beuerle T, Ullmann P, Werck-Reichhart D, Pichersky E (2002) Differential production of meta hydroxylated phenylpropanoids in sweet basil peltate glandular trichomes and leaves is controlled by the activities of specific acyltransferases and hydroxylases. Plant Physiol 130:1536–1544

Goicoechea M, Lacombe E, Legay S, Mihaljevic S, Rech P, Jauneau A, Lapierre C, Pollet B, Verhaegen D, Chaubet-Gigot N, Grima-Pettenati J (2005) Eg-MYB2, a new transcriptional activator from Eucalyptus xylem, regulates secondary cell wall formation and lignin biosynthesis. Plant J 43:553–567

Gopalakrishnan G, Banumathi B, Suresh G (1997) Evaluation of the antifungal activity of natural xanthones from *Garcinia mangostana* and their synthetic derivatives. J Nat Prod 60:519–524

van Gorcom R, Boschloo J, Kuijvenhoven A, Lange J, van Vark A, Bos C, van Balken J, Pouwels P, van den Hondel C (1990) Isolation and molecular characterisation of the benzoate-*para*-hydroxylase gene (bphA) of *Aspergillus niger*: a member of a new gene family of the cytochrome P450 superfamily. Mol Gen Genet 223:192–197

Graf BA, Milbury PE, Blumberg JB (2005) Flavonols, flavones, flavanones, and human health: epidemiological evidence. J Med Food 8:281–290

Grand C (1984) Ferulic acid 5-hydroxylase: a new cytochrome P-450-dependent enzyme from higher plant microsomes involved in lignin synthesis. FEBS Lett 169:7–11

Gravot A, Larbat R, Hehn A, Lievre K, Gontier E, Goergen JL, Bourgaud F (2004) Cinnamic acid 4-hydroxylase mechanism-based inactivation by psoralen derivatives: cloning and characterization of a C4H from a psoralen producing plant—*Ruta graveolens*—exhibiting low sensitivity to psoralen inactivation. Arch Biochem Biophys 422:71–80

Gupta P, Lewis J (1971) Biogenesis of xanthones in *Gentiana lutea*. J Chem Soc C: 629–631

Hahlbrock K, Scheel D (1989) Physiology and molecular biology of phenylpropanoid metabolism. Annu Rev Plant Physiol Plant Mol Biol 40:347–369

Hamada K, Nishida T, Yamauchi K, Fukushima K, Kondo R, Tsutsumi Y (2004) 4-Coumarate:coenzyme A ligase in black locust (*Robinia pseudoacacia*) catalyses the conversion of sinapate to sinapoyl-CoA. J Plant Res 117:303–310

Hamberger B, Hahlbrock K (2004) The 4-coumarate:CoA ligase gene family in *Arabidopsis thaliana* comprises one rare, sinapate-activating and three commonly occurring isoenzymes. Proc Natl Acad Sci USA 101:2209–2214

Hasemann CA, Kurumbail RG, Boddupalli SS, Peterson JA, Deisenhofer J (1995) Structure and function of cytochromes P450: a comparative analysis of three crystal structures. Structure 3:41–62

Hay AE, Helesbeux JJ, Duval O, LabaIed M, Grellier P, Richomme P (2004) Antimalarial xanthones from

Calophyllum caledonicum and *Garcinia vieillardii*. Life Sci 75:3077–3085

Heller W, Kühnl T (1985) Elicitor induction of a microsomal 5-O-(4-coumaroyl)shikimate 3′-hydroxylase in parsley cell suspension cultures. Arch Biochem Biophys 241:453–460

Ho C, Huang Y, Chen C (2002) Garcinone E, a xanthone derivative, has potent cytotoxic effect against hepatocellular carcinoma cell lines. Planta Med 68:975–979

Hotze M, Schröder G, Schröder J (1995) Cinnamate 4-hydroxylase from *Catharanthus roseus* and a strategy for the functional expression of plant cytochrome P450 proteins as translational fusions with P450 reductase in *Escherichia coli*. FEBS Lett 374:345–350

Hübner S, Hehmann M, Schreiner S, Martens S, Lukacin R, Matern U (2003) Functional expression of cinnamate 4-hydroxylase from *Ammi majus* L. Phytochemistry 64:445–452

Humphreys JM, Chapple C (2002) Rewriting the lignin roadmap. Curr Opin Plant Biol 5:224–229

Humphreys JM, Hemm MR, Chapple C (1999) New routes for lignin biosynthesis defined by biochemical characterization of recombinant ferulate 5-hydroxylase, a multifunctional cytochrome P450-dependent monooxygenase. Proc Natl Acad Sci USA 96:10045–10050

Huntley SK, Ellis D, Gilbert M, Chapple C, Mansfield SD (2003) Significant increases in pulping efficiency in C4H-F5H-transformed Poplars: improved chemical savings and reduced environmental toxins. J Agric Food Chem 51:6178–6183

International Rice Genome Sequencing Project (2005) The map-based sequence of the rice genome. Nature 436:793–800

Jensen S, Kjær A, Nielsen B (1975) The genus *Cornus*: non-flavonoid glucosides as taxonomic markers. Biochem Syst Ecol 3:75–78

Jensen SR, Albach DC, Ohno T, Grayer RJ (2005) *Veronica*: iridoids and cornoside as chemosystematic markers. Biochem Syst Ecol 33:1031–1047

Jin H, Cominelli E, Bailey P, Parr A, Mehrtens F, Jones J, Tonelli C, Weisshaar B, Martin C (2000) Transcriptional repression by AtMYB4 controls production of UV-protecting sunscreens in Arabidopsis. EMBO J 19:6150–6161

Kamsteeg J, van Brederode J, Verschuren P, van Nigtevecht G (1981) Identification and genetic control of *p*-coumaroyl-coenzyme A, 3-hydroxylase isolated from petals of *Silene dioica*. Z Pflanzenphysiol 102:435–442

Karpinska B, Karlsson M, Srivastava M, Stenberg A, Schrader J, Sterky F, Bhalerao R, Wingsle G (2004) MYB transcription factors are differentially expressed and regulated during secondary vascular tissue development in hybrid aspen. Plant Mol Biol 56:255–270

Kim YJ, Kim DG, Lee SH, Lee I (2006) Wound-induced expression of the ferulate 5-hydroxylase gene in *Camptotheca acuminata*. Biochim. Biophys Acta 1760:182–190

Kliebenstein DJ, Rowe HC, Denby KJ (2005) Secondary metabolites influence Arabidopsis/Botrytis interactions: variation in host production and pathogen sensitivity. Plant J 44:25–36

Kneusel R, Matern U, Nicolay K (1989) Formation of *trans*-caffeoyl-CoA from *trans*-4-coumaroyl-CoA by Zn^{2+}-dependent enzymes in cultured plant cells and its activation by an elictor-induced pH shift. Arch Biochem Biophys 269:455–462

Koiwai A, Kitano H, Fukuda M, Kisaki T (1974) Methylenedioxyphenyl and its related compounds as protectants against ozone injury to plants. Agric Biol Chem 38:301–307

Kojima M, Takeuchi W (1989) Detection and characterization of *p*-coumaric acid hydroxylase in mung bean, *Vigna mungo*, seedlings. J Biochem 105:265–270

Koopmann E, Logemann E, Hahlbrock K (1999) Regulation and functional expression of cinnamate 4-hydroxylase from parsley. Plant Physiol 119:49–56

Koopmann E, Hahlbrock K (1997) Differentially regulated NADPH:cytochrome P450 oxidoreductases in parsley. Proc Natl Acad Sci USA 94:14954–14959

Kuhlmann S, Kranz K, Lücking B, Alfermann AW, Petersen M (2002) Aspects of cytotoxic lignan biosynthesis in suspension cultures of *Linum nodiflorum*. Phytochem Rev 1:37–43

Kühnl T, Koch U, Heller W, Wellmann E (1987) Chlorogenic acid biosynthesis: characterization of a light-induced microsomal 5-*O*-(4-coumaroyl)-D-quinate/shikimate 3′-hydroxylase from carrot (*Daucus carota* L.) cell suspension cultures. Arch Biochem Biophys 258:226–232

Landry LG, Chapple CCS, Last RL (1995) Arabidopsis mutants lacking phenolic sunscreens exhibit enhanced ultraviolet-B injury and oxidative damage. Plant Physiol 109:1159–1166

Lang D, Eisinger J, Reski R, Rensing S (2005) Representation and high-quality annotation of the *Physcomitrella patens* transcriptome demonstrates a high proportion of proteins involved in metabolism among mosses. Plant Biol 7:238–250

Leon J, Yalpani N, Raskin I, Lawton MA (1993) Induction of benzoic acid 2-hydroxylase in virus-inoculated tobacco. Plant Physiol 103:323–328

Leon J, Shulaev V, Yalpani N, Lawton MA, Raskin I (1995) Benzoic acid 2-hydroxylase, a soluble oxygenase from tobacco, catalyzes salicylic acid biosynthesis. Proc Natl Acad Sci USA 92:10413–10417

Li L, Zhou Y, Cheng X, Sun J, Marita JM, Ralph J, Chiang VL (2003) Combinatorial modification of multiple lignin traits in trees through multigene cotransformation. Proc Natl Acad Sci USA 100:4939–4944

Likhitwitayawuid K, Phadungcharoen T, Krungkrai J (1998) Antimalarial xanthones from *Garcinia cowa*. Planta Med 64:70–72

Lindermayr C, Mollers B, Fliegmann J, Uhlmann A, Lottspeich F, Meimberg H, Ebel J (2002) Divergent members of a soybean (*Glycine max* L.) 4-coumarate:coenzyme. A ligase gene family Primary structures, catalytic properties, and differential expression. Eur J Biochem 269:1304–1315

Logemann E, Parniske M, Hahlbrock K (1995) Modes of expression and common structural features of the

complete phenylalanine ammonia-lyase gene family in parsley. Proc Natl Acad Sci USA 92:5905–5909

Long M, Millar DJ, Kimura Y, Donovan G, Rees J, Fraser PD, Bramley PM, Bolwell GP (2006) Metabolite profiling of carotenoid and phenolic pathways in mutant and transgenic lines of tomato: identification of a high antioxidant fruit line. Phytochem, in press

Löscher R, Heide L (1994) Biosynthesis of *p*-hydroxybenzoate from *p*-coumarate and *p*-coumaroyl-coenzyme A in cell-free extracts of *Lithospermum erythrorhizon* cell cultures. Plant Physiol 106:271–279

Marita JM, Ralph J, Hatfield RD, Chapple C (1999) NMR characterization of lignins in Arabidopsis altered in the activity of ferulate 5-hydroxylase. Proc Natl Acad Sci USA 96:12328–12332

Mason ME, Davis JM (1997) Defense response in Slash pine: chitosan treatment alters the abundance of specific mRNAs. Mol Plant-Microbe Interact 10:135–137

Matsuno M, Nagatsu A, Ogihara Y, Ellis BE, Mizukami H (2002) CYP98A6 from *Lithospermum erythrorhizon* encodes 4-coumaroyl-4′-hydroxyphenyllactic acid 3-hydroxylase involved in rosmarinic acid biosynthesis. FEBS Lett 514:219–224

Matsuzaki F, Wariishi H (2005) Molecular characterization of cytochrome P450 catalyzing hydroxylation of benzoates from the white-rot fungus *Phanerochaete chrysosporium*. Biochem Biophys Res Commun 334:1184–1190

Mauch-Mani B, Slusarenko AJ (1996) Production of salicylic acid precursors is a major function of phenylalanine ammonia-lyase in the resistance of Arabidopsis to *Peronospora parasitica*. Plant Cell 8:203–212

Meuwly P, Molders W, Buchala A, Metraux JP (1995) Local and systemic biosynthesis of salicylic acid in infected cucumber plants. Plant Physiol 109:1107–1114

Meyer K, Cusumano JC, Somerville C, Chapple CC (1996) Ferulate-5-hydroxylase from *Arabidopsis thaliana* defines a new family of cytochrome P450-dependent monooxygenases. Proc Natl Acad Sci USA 93:6869–6874

Meyer K, Shirley AM, Cusumano JC, Bell-Lelong DA, Chapple C (1998) Lignin monomer composition is determined by the expression of a cytochrome P450-dependent monooxygenase in Arabidopsis. Proc Natl Acad Sci USA 95:6619–6623

Mizutani M, Ward E, Dimaio J, Ohta D, Ryals J, Sato R (1993) Molecular cloning and sequencing of a cDNA encoding mung bean cytochrome P450 (P450C4H) possessing cinnamate 4-hydroxylase activity. Biochem Biophys Res Commun 190:875–880

Mizutani M, Ohta D, Sato R (1997) Isolation of a cDNA and a genomic glone encoding cinnamate 4-hydroxylase from Arabidopsis and its expression manner in planta. Plant Physiol 113:755–763

Molog G, Empt U, Kuhlmann S, van Uden W, Pras N, Alfermann A, Petersen M (2001) Deoxypodophyllotoxin 6-hydroxylase, a cytochrome P450 monooxygenase from cell cultures of *Linum flavum* involved in the biosynthesis of cytotoxic lignans. Planta 214:288–294

Momose K, Rudney H (1972) 3-Polyprenyl-4-hydroxybenzoate synthesis in the inner membrane of mitochondria from *p*-hydroxybenzoate and isopentenylpyrophosphate. A demonstration of isoprenoid synthesis in rat liver mitochondria. J Biol Chem 247:3930–3940

Morant M, Hehn A, Werck-Reichhart D (2002) Conservation and diversity of gene families explored using the CODEHOP strategy in higher plants. BMC Plant Biol 2:7

Morant M, Schoch GA, Ullmann P, Ertunç T, Little D, Olsen CE, Petersen M, Negrel J, Werck-Roichhart D (2006) Catalytic activity, duplication and evolution of the CYP98 cytochrome P450 family in wheat. Plant Mol Biol (in press)

Morgenstern B (2004) DIALIGN: multiple DNA and protein sequence alignment at BiBiServ. Nucleic Acids Res 32:W33–36

Nair PM, Vining LC (1965) Cinnamic acid hydroxylase in Spinach. Phytochemistry 4:161–168

Nair RB, Joy RW, IV, Kurylo E, Shi X, Schnaider J, Datla RSS, Keller WA, Selvaraj G (2000) Identification of a CYP84 family of cytochrome P450-dependent monooxygenase genes in *Brassica napus* and perturbation of their expression for engineering sinapine reduction in the seeds. Plant Physiol 123:1623–1634

Nair RB, Xia Q, Kartha CJ, Kurylo E, Hirji RN, Datla R, Selvaraj G (2002) Arabidopsis CYP98A3 mediating aromatic 3-hydroxylation. Developmental regulation of the gene, and expression in yeast. Plant Physiol 130:210–220

Nair RB, Bastress KL, Ruegger MO, Denault JW, Chapple C (2004) The *Arabidopsis thaliana REDUCED EPIDERMAL FLUORESCENCE1* gene encodes an aldehyde dehydrogenase involved in ferulic acid and sinapic acid biosynthesis. Plant Cell 16:544–554

Nedelkina S, Jupe S, Blee K, Schalk M, Werck-Reichhart D, Bolwell GP (1999) Novel characteristics and regulation of a divergent cinnamate 4-hydroxylase (CYP73A15) from french bean: engineering expression in yeast. Plant Mol Biol 39:1079–1090

Ni W, Fahrendorf T, Ballance G, Lamb C, Dixon R (1996) Stress responses in alfalfa (*Medicago sativa* L.). XX. Transcriptional activation of phenlpropanoid pathway genes in elicitor-induced cell suspension cultures. Plant Mol Biol 30:427–438

Ogawa D, Nakajima N, Sano T, Tamaoki M, Aono M, Kubo A, Kanna M, Ioki M, Kamada H, Saji H (2005) Salicylic acid accumulation under O3 exposure is regulated by ethylene in tobacco plants. Plant Cell Physiol 46:1062–1072

Ohe T, Mashino T, Hirobe M (1997) Substituent elimination from *p*-substituted phenols by cytochrome. P450 ipso-substitution by the oxygen atom of the active species. Drug Metab Dispos 25:116–122

Omer C, Lenstra R, Litle P, Dean C, Tepperman J, Leto K, Romesser J, O'Keefe D (1990) Genes for two

herbicide-inducible cytochromes P-450 from *Streptomyces griseolus*. J Bacteriol 172:3335–33445
Osakabe K, Tsao CC, Li L, Popko JL, Umezawa T, Carraway DT, Smeltzer RH, Joshi CP, Chiang VL (1999) Coniferyl aldehyde 5-hydroxylation and methylation direct syringyl lignin biosynthesis in angiosperms. Proc Natl Acad Sci USA 96:8955–8960
Overkamp S, Hein F, Barz W (2000) Cloning and characterization of eight cytochrome P450 cDNAs from chickpea (*Cicer arietinum* L.) cell suspension cultures. Plant Sci 155:101–108
Panossian A, Wagner H (2005) Stimulating effect of adaptogens: an overview with particular reference to their efficacy following single dose administration. Phytother Res 19:819–838
Pasqualini S, la Torre G, Ferranti F, Ederli L, Piccioni C, Reale L, Antonielli M (2002) Salicylic acid modulates ozone-induced hypersensitive cell death in tobacco plants. Physiol Plant 115:204–212
Patzlaff A, McInnis S, Courtenay A, Surman C, Newman LJ, Smith C, Bevan MW, Mansfield S, Whetten RW, Sederoff RR, Campbell MM (2003) Characterisation of a pine MYB that regulates lignification. Plant J 36:743–754
Peres V, Nagem TJ, de Oliveira FF (2000) Tetraoxygenated naturally occurring xanthones. Phytochemistry 55:683–710
Peters S, Schmidt W, Beerhues L (1997) Regioselective oxidative phenol couplings of 2,3′,4,6-tetrahydroxybenzophenone in cell cultures of *Centaurium erythraea* RAFN and *Hypericum androsaemum* L. Planta 204:64–69
Petersen M (1997) Cytochrome P450-dependent hydroxylation in the biosynthesis of rosmarinic acid in Coleus. Phytochemistry 45:1165–1172
Petersen M (2003) Cinnamic acid 4-hydroxylase from cell cultures of the hornwort *Anthoceros agrestis*. Planta 217:96–101
Pi Y, Liao Z, Chai Y, Zeng H, Wang P, Gong Y, Pang Y, Sun X, Tang K (2006) Molecular cloning and characterization of a novel stem-specific gene from *Camptotheca acuminata*. J Biochem Mol Biol 39:68–75
Pierrel MA, Batard Y, Kazmaier M, Mignotte-Vieux C, Durst F, Werck-Reichhart D (1994) Catalytic properties of the plant cytochrome P450 CYP73 expressed in yeast. Substrate specificity of a cinnamate hydroxylase. Eur J Biochem 224:835–844
Raes J, Rohde A, Christensen JH, Van de Peer Y, Boerjan W (2003) Genome-wide characterization of the lignification toolbox in Arabidopsis. Plant Physiol 133:1051–1071
Ralph J, Akiyama T, Kim H, Lu F, Schatz PF, Marita JM, Ralph SA, Srinivasa Reddy MS, Chen F, Dixon RA (2006) Effects of coumarate 3-hydroxylase down-regulation on lignin structure. J Biol Chem 281:8843–8853
Ralston L, Kwon ST, Schoenbeck M, Ralston J, Schenk DJ, Coates RM, Chappell J (2001) Cloning, heterologous expression, and functional characterization of 5-epi-aristolochene-1,3-dihydroxylase from tobacco (*Nicotiana tabacum*). Arch Biochem Biophys 393:222–235
Ralston L, Yu O (2006) Metabolons involving plant cytochrome P450s. Phytochem Rev, this issue
Reddy MSS, Chen F, Shadle G, Jackson L, Aljoe H, Dixon RA (2005) Targeted down-regulation of cytochrome P450 enzymes for forage quality improvement in alfalfa (*Medicago sativa* L.). Proc Natl Acad Sci USA 102:16573–16578
Ro DK, Mah N, Ellis BE, Douglas CJ (2001) Functional characterization and subcellular localization of poplar (*Populus trichocarpa* × *Populus deltoides*) cinnamate 4-hydroxylase. Plant Physiol 126:317–329
Rogers LA, Campbell MM (2004) The genetic control of lignin deposition during plant growth and development. New Phytol 164:17–30
Rogers LA, Dubos C, Cullis IF, Surman C, Poole M, Willment J, Mansfield SD, Campbell MM (2005) Light, the circadian clock, and sugar perception in the control of lignin biosynthesis. J Exp Bot 56:1651–1663
Ruegger M, Meyer K, Cusumano JC, Chapple C (1999) Regulation of ferulate-5-hydroxylase expression in Arabidopsis in the context of sinapate ester biosynthesis. Plant Physiol 119:101–110
Ruegger M, Chapple C (2001) Mutations that reduce sinapoylmalate accumulation in *Arabidopsis thaliana* define loci with diverse roles in phenylpropanoid metabolism. Genetics 159:1741–1749
Russel DW, Conn EE (1967) The cinnamic acid 4-hydroxylase of pea seedlings. Arch Biochem Biophys 122:256–258
Saleem M, Kim H, Ali M, Lee Y (2005) An update on bioactive plant lignans. Nat Prod Rep 22:696–716
Schmidt W, Beerhues L (1997) Alternative pathways of xanthone biosynthesis in cell cultures of *Hypericum androsaemum* L. FEBS Lett 420:143–146
Schmidt W, Peters S, Beerhues L (2000) Xanthone 6-hydroxylase from cell cultures of *Centaurium erythraea* RAFN and *Hypericum androsaemum* L. Phytochemistry 53:427–431
Schnitzler JR-P, Madlung J, Rose A, Seitz UH (1992) Biosynthesis of *p*-hydroxybenzoic acid in elicitor-treated carrot cell cultures. Planta 188:594–600
Schoch G, Goepfert S, Morant M, Hehn A, Meyer D, Ullmann P, Werck-Reichhart D (2001) CYP98A3 from *Arabidopsis thaliana* is a 3′-hydroxylase of phenolic esters, a missing link in the phenylpropanoid pathway. J Biol Chem 276:36566–36574
Schoch GA, Nikov GN, Alworth WL, Werck-Reichhart D (2002) Chemical inactivation of the cinnamate 4-hydroxylase allows for the accumulation of salicylic acid in elicited cells. Plant Physiol 130:1022–1031
Schopfer CR, Ebel J (1998) Identification of elicitor-induced cytochrome P450s of soybean (*Glycine max* L.) using differential display of mRNA. Mol Gen Genom 258:315–322
Sewalt VJH, Ni W, Blount JW, Jung HG, Masoud SA, Howles PA, Lamb C, Dixon RA (1997) Reduced lignin content and altered lignin composition in transgenic tobacco down-regulated in expression of

L-phenylalanine ammonia-lyase or cinnamate 4-hydroxylase. Plant Physiol 115:41–50

Shimoda K, Yamane, S-Y, Hirakawa H, Ohta S, Hirata T (2002) Biotransformation of phenolic compounds by the cultured cells of *Catharanthus roseus*. J Mol Catal B: Enzym 16:275–281

Shimoda K, Kubota N, Sano T, Hirakawa H, Hirata T (2004) A novel hydroxylase from *Catharanthus roseus* participating in the hydroxylation of 2-hydroxybenzoic acid. J Biosci Bioeng 98:67–70

Sibout R, Baucher M, Gatineau M, Van Doorsselaere J, Mila I, Pollet B, Maba B, Pilate G, Lapierre C, Boerjan W, Jouanin L (2002) Expression of a poplar cDNA encoding a ferulate-5-hydroxylase/coniferaldehyde 5-hydroxylase increases S lignin deposition in *Arabidopsis thaliana*. Plant Physiol Biochem 40:1087–1096

Stafford H, Dresler S (1972) 4-Hydroxycinnamic acid hydroxylase and polyphenolase activities in *Sorghum vulgare*. Plant Physiol 49:590–595

Tamagnone L, Merida A, Parr A, Mackay S, Culianez-Macia FA, Roberts K, Martin C (1998) The AmMYB308 and AmMYB330 transcription factors from Antirrhinum regulate phenylpropanoid and lignin biosynthesis in transgenic tobacco. Plant Cell 10:135–154

Tanaka Y (2006) Flower colour and cytochromes P450. Phytochem Rev, this issue

Taskova RM, Gotfredsen CH, Jensen SR (2005) Chemotaxonomic markers in Digitalideae (Plantaginaceae). Phytochemistry 66:1440–1447

Teutsch HG, Hasenfratz MP, Lesot A, Stoltz C, Garnier J, Jeltsch J, Durst F, Werck-Reichhart D (1993) Isolation and sequence of a cDNA encoding the Jerusalem artichoke cinnamate 4-hydroxylase, a major plant cytochrome P450 involved in the general phenylpropanoid pathway. Proc Natl Acad Sci USA 90:4102–4106

Urban P, Werck-Reichhart D, Teutsch HG, Durst F, Regnier S, Kazmaier M, Pompon D (1994) Characterization of recombinant plant cinnamate 4-hydroxylase produced in yeast. Kinetic and spectral properties of the major plant P450 of the phenylpropanoid pathway. Eur J Biochem 222:843–850

Urban P, Mignotte C, Kazmaier M, Delorme F, Pompon D (1997) Cloning, yeast expression, and characterization of the coupling of two distantly related *Arabidopsis thaliana* NADPH-cytochrome P450 reductases with P450 CYP73A5. J Biol Chem 272:19176–19186

Vaughan PF, Butt VS (1970) The action of o-dihydric phenols in the hydroxylation of *p*-coumaric acid by a phenolase from leaves of spinach beet (*Beta vulgaris* L.). Biochem J 119:89–94

Vlietinck A, De Bruyne T, Apers S, Pieters L (1998) Plant-derived leading compounds for chemotherapy of human immunodeficiency virus (HIV) infection. Planta Med 64:97–109

Wang ZX, Li SM, Loscher R, Heide L (1997) 4-Coumaroyl coenzyme A 3-hydroxylase activity from cell Cultures of *Lithospermum erythrorhizon* and its relationship to polyphenol oxidase. Arch Biochem Biophys 347:249–255

Wang CZ, Maier UH, Keil M, Zenk MH, Bacher A, Rohdich F, Eisenreich W (2003) Phenylalanine-independent biosynthesis of 1,3,5,8-tetrahydroxyxanthone. A retrobiosynthetic NMR study with root cultures of *Swertia chirata*. Eur J Biochem 270:2950–2958

Weisshaar B, Jenkins GI (1998) Phenylpropanoid biosynthesis and its regulation. Curr Opin Plant Biol 1:251–257

Werck-Reichhart D, Batard Y, Kochs G, Lesot A, Durst F (1993) Monospecific polyclonal antibodies directed against purified cinnamate 4-hydroxylase from *Helianthus tuberosus* (immunopurification, immunoquantitation, and interspecies cross-reactivity). Plant Physiol 102:1291–1298

Werck-Reichhart D (1995) Cytochromes P450 in phenylpropanoid metabolism. Drug Metabol Drug Interact 12:221–243

Whitbred JM, Schuler MA (2000) Molecular characterization of CYP73A9 and CYP82A1 P450 genes involved in plant defense in pea. Plant Physiol 124:47–58

Wildermuth MC, Dewdney J, Wu G, Ausubel FM (2001) Isochorismate synthase is required to synthesize salicylic acid for plant defence. Nature 414:562–565

Wu S, Zu Y, Wu M (2003) High yield production of salidroside in the suspension culture of *Rhodiola sachalinensis*. J Biotech 106:33–43

Yalpani N, Leon J, Lawton MA, Raskin I (1993) Pathway of salicylic acid biosynthesis in healthy and virus-inoculated tobacco. Plant Physiol 103:315–321

Yamamoto H, Yoshida K, Kondo Y, Inoue K (1998) Production of cornoside in *Abeliophyllum distichum* cell suspension cultures. Phytochemistry 48:273–277

Yamamoto H, Inoue K, Li SM, Heide L (2000) Geranylhydroquinone 3′-hydroxylase, a cytochrome P-450 monooxygenase from *Lithospermum erythrorhizon* cell suspension cultures. Planta 210:312–317

Yamamoto H, Hori M, Kuwajima H, Inoue K (2003) Formation of benzoquinol moiety in cornoside by salidroside mono-oxygenase, a cytochrome P450 enzyme, from *Abeliophyllum distichum* cell suspension cultures. Planta 216:432–436

Yamauchi K, Yasuda S, Hamada K, Tsutsumi Y, Fukushima K (2003) Multiform biosynthetic pathway of syringyl lignin in angiosperms. Planta 216:496–501

Ye Z-H (1996) Expression patterns of the cinnamic acid 4-hydroxylase gene during lignification in *Zinnia elegans*. Plant Sci 121:133–141