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CYP98A3 from *Arabidopsis thaliana* Is a 3'-Hydroxylase of Phenolic Esters, a Missing Link in the Phenylpropanoid Pathway*

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The 4- and 5-hydroxylations of phenolic compounds in plants are catalyzed by cytochrome P450 enzymes. The 3-hydroxylation step leading to the formation of caffeic acid from *p*-coumaric acid remained elusive, however, alternatively described as a phenol oxidase, a dioxygenase, or a P450 enzyme, with no decisive evidence for the involvement of any in the reaction *in planta*. In this study, we show that the gene encoding CYP98A3, which was the best possible P450 candidate for a 3-hydroxylase in the *Arabidopsis* genome, is highly expressed in inflorescence stems and wounded tissues. Recombinant CYP98A3 expressed in yeast did not metabolize free *p*-coumaric acid or its glucose or CoA esters, *p*-coumaraldehyde, or *p*-coumaryl alcohol, but very actively converted the 5-*O*-shikimate and 5-*O*-*D*-quinic acid esters of *trans-p*-coumaric acid into the corresponding caffeic acid conjugates. The shikimate ester was converted four times faster than the quinic derivative. Antibodies directed against recombinant CYP98A3 specifically revealed differentiating vascular tissues in stem and root. Taken together, these data show that CYP98A3 catalyzes the synthesis of chlorogenic acid and very likely also the 3-hydroxylation of lignin monomers. This hydroxylation occurs on depsides, the function of which was so far not understood, revealing an additional and unexpected level of networking in lignin biosynthesis.

Systematic genome sequencing is revealing a large number of orphan genes and their phylogenetic relatedness to genes with characterized function. EST¹ sequences, on the other hand, are providing preliminary information on levels, patterns of expression, and conservation of genes among species. Taken together, such information can be exploited as a clue to gene function and to track down missing steps in important pathways.

The sequencing of the whole genome of *Arabidopsis thaliana* has revealed 273 cytochrome P450 genes distributed into 45 families and subfamilies (drnelson.utmem.edu/CytochromeP450.html, www.biobase.dk/P450/). P450 proteins thus form the largest superfamily of enzymes involved in plant metabolism, but the

function of 80% of these enzymes is still unknown. Our attention was first drawn to the CYP98 family by its phylogeny and structure. An analysis of P450 phylogeny in *A. thaliana* (Fig. 1) shows that the CYP98 family is most closely related to CYP73A5, coding for the cinnamic-acid 4-hydroxylase, the second enzyme and first P450 in the phenylpropanoid pathway (1). CYP73A5 and the CYP98 family seem to have evolved from the same ancestor as CYP79 enzymes, some of which also, in common with CYP73A5, use aromatic substrates derived from the shikimate pathway (2, 3). It was thus tempting to speculate that the substrate of CYP98 enzymes was derived from aromatic amino acids as well. The *Arabidopsis* CYP98 family is formed by only three genes. CYP98A3 is present in single copy; CYP98A8 and CYP98A9 are 69% identical to one another and only 52% identical to CYP98A3. All P450 genes in the phenylpropanoid pathway (CYP73A5, CYP84A1, and CYP75B1) that have been characterized so far in *Arabidopsis* are present in single copy in the genome. Such a situation is unusual in other P450 families, with most of them showing multiple duplications. Phylogenetic analysis thus pointed to CYP98A3 as an enzyme likely to be involved in the phenylpropanoid pathway.

Such a hypothesis was supported by the high frequency of CYP98A3 ESTs reported in many *Arabidopsis* libraries (root, rosette, inflorescence, silique, seed), but also by the high frequency of other CYP98 ESTs detected in a variety of plants species and tissues. Among tissues expressing high levels of CYP98 message were poplar and pine xylem (4, 5), soybean hypocotyl and stem, as well as cotton fibers. In support of the latter EST data, a CYP98 cDNA was PCR-isolated from sweet gum xylem together with those for CYP73 and CYP84, which catalyze cinnamic acid and coniferylaldehyde hydroxylations in lignin biosynthesis (6). Message frequency, wide distribution, and location thus suggested probable involvement of CYP98A3 enzymes in a high throughput pathway and a function in the formation of some structural element, possibly the formation or reinforcement of the cell wall. A good candidate function for CYP98A3 was 3-hydroxylation of the phenylpropanoid ring, a still elusive step in the phenylpropanoid pathway needed for the synthesis of lignin monomers and other abundant and widespread plant compounds such as chlorogenic acid.

In this study, we confirm by RNA blotting that CYP98A3 is constitutively expressed in all plant tissues and show that its message accumulation is increased in wounded leaves. The CYP98A3 protein expressed in yeast does not metabolize free *p*-coumaric acid or its glucose or CoA esters, but hydroxylates the coumaroyl esters of shikimic and quinic acids with a high efficiency, higher than previously reported for the 4-hydroxylation of cinnamic acid, the upstream P450-catalyzed step in the phenylpropanoid pathway. The enzyme selectively metabolizes the natural 5-*O*- and *trans*-isomers of the substrates. Poly-

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¹ The abbreviations used are: EST, expressed sequence tag; PCR, polymerase chain reaction; HPLC, high pressure liquid chromatography; C₃H, *p*-coumaroyl-shikimate/quinic acid 3'-hydroxylase; CCoAOMT, caffeoyl-CoA *O*-methyltransferase.

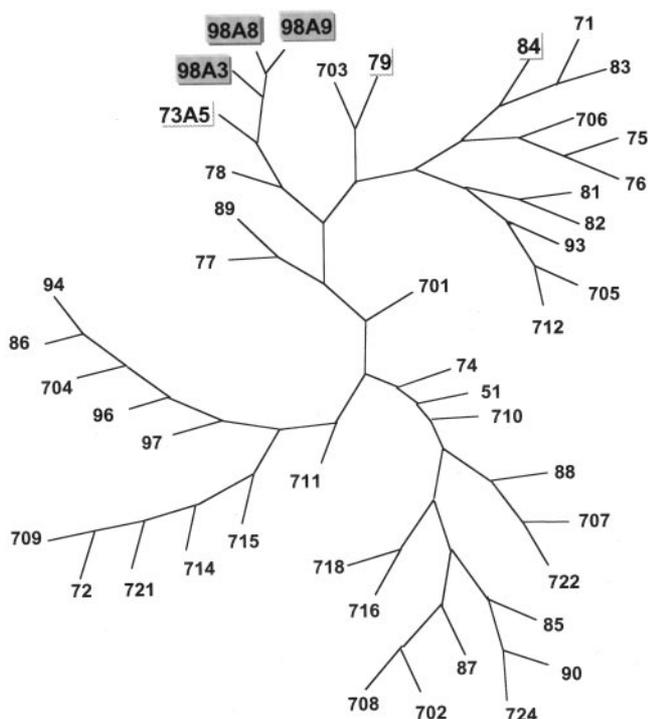


FIG. 1. Phylogenetic tree of cytochromes P450 from *A. thaliana*. For simplification, the tree was built using consensus sequences representative of each family, except for CYP98 enzymes, which are all represented. Families known to metabolize aromatic amino acids or simple phenolic compounds are shaded. The tree is unrooted and does not show distances.

clonal antibodies raised against the recombinant enzyme specifically label lignin-synthesizing tissues in stem and roots. Taken together, these data demonstrate that CYP98A3 is involved in the biosynthesis of chlorogenic acid and strongly suggest that it also catalyzes the 3-hydroxylation of the lignin monomers. Although previously foreseen by Heller and Kühnl (7), this new development is rather unexpected and raises a new degree of complexity and additional gridding level in the already complex lignin biosynthesis pathway.

EXPERIMENTAL PROCEDURES

Chemicals—Chlorogenic, shikimic, D-quinic, *p*-coumaric, caffeic, and ferulic acids were from Sigma (l'Isle d'Abeau Chesnes, France). *p*-Coumaryl alcohol and *p*-coumaraldehyde were gifts from Dr. M. Barber (Southampton University). *trans*-5-*O*-Coumaroyl- and *trans*-5-*O*-caffeoylshikimic acids were gifts from Dr. W. Heller (Forschungszentrum für Umwelt und Gesundheit, Munich, Germany). β -Megaspermin was provided by Dr. S. Kauffmann (Institute of Plant Molecular Biology, Strasbourg, France). *p*-[¹⁴C]Coumaric acid was synthesized enzymatically from *trans*-[3-¹⁴C]cinnamate (Isotopchim, Ganagobie, France) using microsomes from recombinant yeast expressing CYP73A1 (8). The 4- and 3-isomers of *p*-coumaroylshikimic acid were generated by heating a solution of the *trans*-5-*O*-isomer in 0.1 M sodium phosphate buffer (pH 7.4) for 1 h at 90 °C, and the *cis*-isomer of *trans*-5-*O*-*p*-coumaroylshikimic acid was obtained by irradiation for 10 min at 254 nm (9). *p*-Cinnamoyl-CoA and *p*-coumaroyl-CoA were synthesized as described (10, 11). Radiolabeled *p*-coumaroyl 1- and 4-glucosides were synthesized using recombinant tobacco glucosyltransferase incubated with *p*-coumaric acid and UDP-[¹⁴C]glucose as described (12).

Cell Culture and Extraction— β -Megaspermin (50 nM) was added under sterile conditions to a flask containing 10 ml of a 6-day-old culture of tobacco Bright Yellow cell suspension. After a 4-h incubation in the dark, cells were harvested by filtration, frozen in liquid nitrogen, and stored at -80 °C. Crude extract was prepared using the protocol described by Heller and Kühnl (7), slightly modified as follows. Four grams of frozen cells were homogenized in a mortar with 0.2 g of Dowex 1 X2 and suspended in 0.1 M potassium phosphate (pH 7) containing 1% polyvinylpyrrolidone, 28 mM 2-mercaptoethanol, 0.2 mM phenylmethylsulfonyl fluoride, and 20 mM sodium metabisulfite. After centrifugation

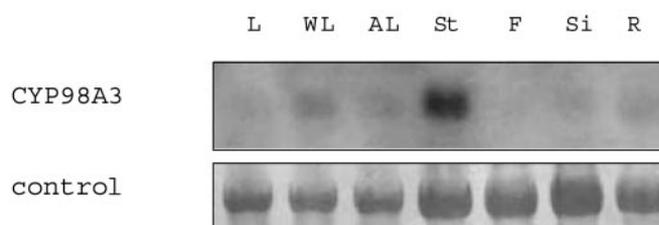


FIG. 2. Tissue distribution of CYP98A3 transcripts in the adult *A. thaliana* plant. Sixteen micrograms of total RNA were loaded in each lane (upper panel). The full-length CYP98A3 sequence was used as a probe. L, leaves; WL, wounded leaves, *i.e.* detached, sliced with a razor blade, and aged 24 h on Murashige and Skoog medium; AL, leaves detached and aged 24 h on Murashige and Skoog medium; St, inflorescence stems; F, flowers; Si, siliques; R, roots. The lower panel shows the methylene blue staining of the membrane to check for loading and transfer efficiency.

at 10,000 \times *g* for 20 min, the supernatant was desalted on a Sephadex G-25 column (HiTrap desalting, Amersham Pharmacia Biotech) equilibrated in 50 mM potassium phosphate (pH 7.0) and 10 mM dithiothreitol. The eluted fraction was concentrated on a Centricon-10 (Amicon, Inc.) and either used directly as a source of hydroxycinnamoyl-CoA: quinate/shikimate hydroxycinnamoyltransferase or stored at -80 °C in 20% glycerol.

Enzymatic Preparation of *p*-Coumaroylquinic Acid—*trans*-5-*O*-*p*-Coumaroyl D-quinic acid was synthesized enzymatically from *trans*-4-coumaroyl-CoA and D-quinic acid using the desalted and concentrated extract from tobacco BY cells. The incubation mixture (containing, in a final volume of 500 μ l, 0.5 mM 4-coumaroyl-CoA, 4 mM D-quinic acid, 50 mM potassium phosphate (pH 7.0), and 250 μ l of the crude extract (0.55 mg of protein) was incubated for 2 h at 28 °C in the dark. After addition of 60 μ l of acetic acid, *p*-coumaroylquinic acid was extracted three times with 1 volume of ethyl acetate and evaporated to dryness under argon. Identity of the product was checked by UV spectroscopy and negative electrospray mass spectrometry (*m/z* 337.3). It was further purified by HPLC for enzyme kinetic analysis.

cDNA Isolation and Expression in Yeast—The CYP98A3 coding sequence was amplified from an *A. thaliana Col-0* silique cDNA library (13) by PCR using primers 5'-CGGGATCCATGTCGTGGTTTCTAAT-AGC and 5'-GCGAATTCTTACATATCGTAAGGCACGC, designed according to the data from genome sequencing (AC002409, T20B5.9). *Bam*HI and *Eco*RI restriction sites were added 5' and 3', respectively, for cloning in the yeast expression vector pYeDP60 (14). The PCR mixture contained 10 ng of template, 20 pmol of primers, 0.5 μ M dNTPs, 3.5 mM MgCl₂, and 10% Me₂SO in a total volume of 50 μ l. It was preheated for 2 min at 94 °C before addition of 5 units of *Pfu* DNA polymerase (Stratagene). After 3 min of additional heating at 94 °C, 25 cycles of amplification were carried out as follows: 1-min denaturation at 92 °C, 30-s annealing at 52 °C, and 5-min extension at 72 °C. The reaction was completed by a 10-min extension at 72 °C plus an additional 30 s at 72 °C after addition of *Taq* DNA polymerase (Life Technologies, Inc.) to graft 3'-A overhangs for cloning into a T-tailed vector. Double-stranded pGEM-T subclones (Promega) were checked by sequencing using the Prism Ready Reaction Dye Deoxy Terminator Cycle method (Applied Biosystems, Inc.) and transferred into expression vectors. The sequence data were analyzed using the GCG Sequence Analysis Software Package (Version 8.1) or ClustalX. Transformation of the *Saccharomyces cerevisiae* strain WAT11, engineered to inducibly express the NADPH-cytochrome P450 reductase from *A. thaliana* ATR1 upon galactose induction, was performed as described (14). Yeast cells were grown as previously described (14), and microsomes were isolated after 16–24 h of induction on 20 g/liter galactose at 20 or 30 °C. Another recombinant yeast strain was constructed, expressing 4-His-tagged CYP98A3, using the 3'-primer 5'-GCGAATTCTTAAATGATGATGATGAT-CATATCGTAAGGCACGCCTT.

The CYP98A8 (AC011765, F1M20.22) and CYP98A9 (AC011765, F1M20.23) coding sequences were amplified by PCR using, as template, *A. thaliana Col-0* genomic DNA with primers 5'-GGAAGATCTATGAT-TATATATCTAATTTTC and 5'-GGGGTACCTTAATCTAAAGGTAAAG-GTA and primers 5'-GGAAGATCTATGATTTTATTACTCATATC and 5'-GCGAATTCTTAAAGGTATAACTCTTGTG, respectively. CYP98A8 was cloned into the *Bam*HI and *Kpn*I sites, and CYP98A9 into the *Bam*HI and *Eco*RI sites of pYeDP60.

Enzyme Purification and Production of Polyclonal Antibodies—CYP98A3, 4-His-tagged at the C terminus, was solubilized in 0.9%

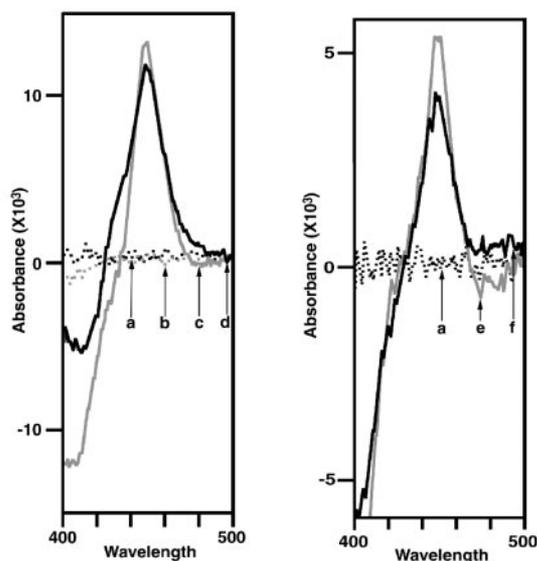


FIG. 3. Expression of CYP98A3, 4-His-tagged CYP98A3, CYP98A8, and CYP98A9 in yeast. Upper panels, CO/reduced difference spectra recorded with microsomes of recombinant yeast transformed with a void plasmid (b) or expressing CYP98A3 (c), 4-His-tagged CYP98A3 (d), CYP98A8 (e), or CYP98A9 (f). a, base line recorded before CO saturation of the assay cuvette. Microsomes are 0.7 mg·ml⁻¹ in the cuvettes. Lower panel, immunoblot analysis of the recombinant microsomes and of an *Arabidopsis* stem crude protein extract with polyclonal antiserum (1/10,000) raised against 4-His-tagged CYP98A3. Six μ g of protein were loaded in each lane. Void, microsomes of yeast transformed with a void plasmid; A3, recombinant CYP98A3; A3H, recombinant CYP98A3 with a 4-His tag; A9, recombinant CYP98A9; A8, recombinant CYP98A8; 73, recombinant CYP73A1 (C4H); stem, crude extract from *Arabidopsis* stem; M, molecular mass markers.

Emulgen 911 (Kao Atlas) and purified on a Ni²⁺-loaded HiTrap chelating column (Amersham Pharmacia Biotech) using the procedure recommended by the manufacturer, with elution in 50 mM sodium phosphate buffer (pH 7.4) containing 10% glycerol, 0.02% Emulgen 911, 0.5 M NaCl, and 60 mM imidazole. Polyclonal antibodies were raised in rabbits by successive injections of 16 μ g (once) and 8 μ g (five times) of purified protein emulsified in Freund's complete and incomplete adjuvants, respectively, and used for Western blot analysis as described (15).

Standard Assay of the 3'-Hydroxylase Activities—The standard assay for *p*-coumaroyl-shikimate/quininate 3'-hydroxylase (C3'H) contained, in a total volume of 200 μ l, 100 mM sodium phosphate buffer (pH 7.4), 3 μ g of microsomal protein from yeast (*i.e.* 0.6 pmol of CYP98A3), 4–145 μ M substrate, and 600 μ M NADPH. The reaction was incubated at 28 °C for 5 min and terminated by addition of 20 μ l of acetic acid. The products were extracted three times with 2 volumes of ethyl acetate; the organic phase was pooled and evaporated under argon; and the residue dissolved in 300 μ l of 10% acetonitrile, 90% water, and 0.2% acetic acid (v/v/v) was analyzed by reverse-phase HPLC (Merck LiChrosorb RP-18 column, 4 \times 125 mm, 5 μ m; flow rate of 1 ml/min; 5 min of isocratic 10% acetonitrile and then a 20-min linear gradient from 10 to 52% acetonitrile in water containing 0.2% acetic acid). For an accurate determination of kinetic constants with the quinate esters that are not efficiently extracted with ethyl acetate, the reaction was stopped with 40% acetonitrile and 0.2% acetic acid before centrifugation for 15 min at 18,000 \times g and 4-fold dilution for HPLC analysis.

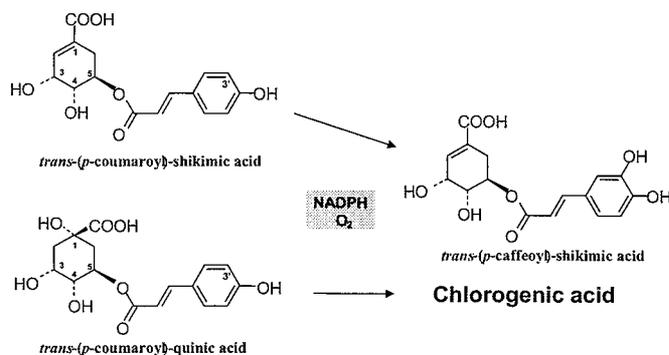


FIG. 4. Reactions catalyzed by C3'H.

Absorbance of the eluant was monitored with a diode array detector. The retention times were 6.5 min for caffeoyl quinate, 10.9 min for caffeoyl shikimate, 10.5 min for coumaroyl quinate, and 12.6 min for coumaroyl shikimate. Substrate conversion was calculated from peak areas at 320 nm by comparison with injected standards. Amounts of substrates and products in incubation media and pooled extracts were calculated using the following extinction coefficients at 340 nm: 6,200 M⁻¹ cm⁻¹ for substrates and 15,700 M⁻¹ cm⁻¹ for products (9). For characterization of the reaction products, HPLC elutions corresponding to the peaks of products were pooled, evaporated, and submitted to mass spectrometry analysis on a BioQ triple quadrupole (Micromass). Kinetic data were fitted using the nonlinear regression program DNRPEASY derived by Duggleby (16) from DNRPF53.

Assay of CoA and Glucose Ester Hydroxylation—Incubations with cinnamoyl-CoA, *p*-coumaroyl-CoA, *p*-coumaroyl glucose ester, and *p*-coumaroyl 4-glucoside were performed as described for the shikimate and quinate esters, except that up to 10-fold higher concentrations of yeast microsomes and longer incubation times were also assayed to exclude any possibility of low rate metabolism. Products, extracted 3-fold with 2 volumes of ethyl acetate, were analyzed both directly and after 1 h of hydrolysis in 1 N HCl at 90 °C. The products of hydrolysis and glucose conjugates were analyzed by HPLC as described for the shikimate and quinate esters, using diode array and radiodetection (Packard Flow Scintillation Analyzer 500TR). HPLC analysis of intact CoA conjugates was performed without prior extraction using a gradient of acetonitrile in 15 mM (NH₄)₂HPO₄ and 15 mM HCl (pH 5.5) (flow rate of 1 ml/min and 3 min of isocratic 10% acetonitrile and then a 13-min linear gradient from 10 to 50% acetonitrile).

Measurement of O-Methyltransferase Activity—Methylation of the C3'H reaction products was assayed using recombinant caffeoyl-CoA O-methyltransferase (CCoAOMT1 from tobacco) expressed in *Escherichia coli* and purified as described (17), alone or combined with hydroxycinnamoyl-CoA transferase prepared from tobacco cells as described above. The assay contained, in 100 μ l of 40 μ M sodium phosphate buffer (pH 7.4), 40 μ M S-adenosylmethionine, 0.2 mM MgCl₂, 2 mM dithiothreitol, 40 μ M 5-O-caffeoyl shikimate or quinate (or caffeoyl-CoA), and 20 μ g of purified protein. In some cases, 22 μ g of tobacco Bright Yellow cell crude extract were added as a source of hydroxycinnamoyl-CoA transferase. After a 2-h incubation at 30 °C, the reaction was stopped by addition of 25 μ l of 4 N HCl. Products were hydrolyzed for 30 min at 90 °C and extracted three times with 2 volumes of ethyl acetate. The organic phase was pooled and evaporated, and the residue dissolved in 300 μ l of 10% acetonitrile, 90% water, and 0.2% acetic acid (v/v/v) was analyzed by reverse-phase HPLC as described above.

Spectrophotometric Measurements—Spectrophotometric measurements of total P450 content (18) and evaluation of substrate binding (19) were performed as described. Substrate binding spectra were recorded using double cuvettes.

RNA Blot Analysis—RNA was isolated from 3-month-old plants. For wounding experiments, leaves were lacerated with a razor blade and aged for 24 h under continuous light in standard Murashige and Skoog medium. Control leaves were aged without laceration. Total RNA was prepared using the RNeasy[®] plant mini-kit (QIAGEN) and quantified, and concentrations were adjusted to 8 μ g/ml. RNA blot analysis was performed using 16 μ g of total RNA separated on a 1.2% denaturing formaldehyde-agarose gel and blotted onto BrightStar Plus[™] membrane (Ambion Inc.). After RNA fixation for 1 h at 80 °C, the membrane was stained with methylene blue to check integrity and equal loading of RNA. The ³²P-labeled probe corresponding to the entire coding region of CYP98A3 was synthesized by random priming using Ready-To-Go[™] DNA labeling beads (Amersham Pharmacia Biotech). The membrane

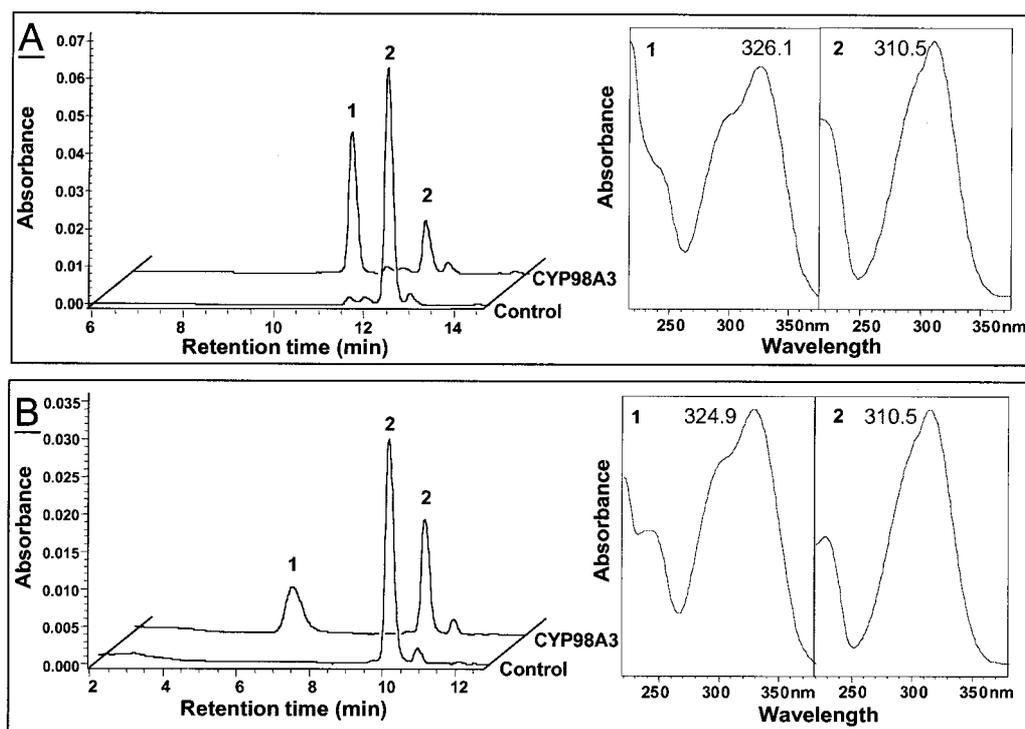


FIG. 5. HPLC analysis of the products of *trans*-5-*O*-(4-coumaroyl)-shikimate and *trans*-5-*O*-(4-coumaroyl)-D-quininate metabolism by recombinant CYP98A3. Absorbance was monitored at 320 nm. Conversion is shown after a 5-min incubation of 2.5 μ mol of recombinant CYP98A3 in a 200- μ l assay. Controls performed in the absence of NADPH or using microsomes of yeast transformed with a void plasmid gave similar results. A, conversion of *trans*-5-*O*-(4-coumaroyl) shikimate (4 nmol in the assay); B, conversion of *trans*-5-*O*-(4-coumaroyl) D-quininate (2 nmol in the assay). Peak 1 is the product, and peak 2 is the substrate. UV spectra are shown on the right.

TABLE I
Catalytic parameters of the 3'-hydroxylation catalyzed by recombinant CYP98A3

Catalytic parameters were determined for the recombinant enzyme coexpressed with the *A. thaliana* P450 reductase ATR1 in yeast microsomes.

Substrate	K_m μ M	K_{cat} min^{-1}	K_{cat}/K_m $min^{-1} \mu$ M $^{-1}$
5- <i>O</i> -(4-Coumaroyl) shikimate	7 ± 1	612 ± 30	87
5- <i>O</i> -(4-Coumaroyl) quininate	18 ± 2	399 ± 19	22

was hybridized in 5 \times SSC, 5 \times Denhardt's solution, 0.5% SDS, 2 mM EDTA, and 100 μ g/ μ l sonicated salmon sperm at 65 $^{\circ}$ C and then washed twice at 60 $^{\circ}$ C in 0.2 \times SSC and 0.1% SDS, and signal was recorded by autoradiography.

Tissue Print Hybridization and Histochemical Detection of Lignin—Stem and root transversal hand cuts were printed onto Schleicher & Schuell 0.2- μ m nitrocellulose, washed twice for 20 min with phosphate-buffered saline containing 0.4% Tween 20, and then blocked and revealed as a standard immunoblot using preimmune or anti-CYP98A3 polyclonal serum diluted 1:1000. Before dilution, the crude serum was incubated for 5 min with an equal volume of microsomes from recombinant yeast overexpressing CYP73A1 (8) to minimize background staining and possible cross-recognition of CYP73 epitopes. Protein-antibody complexes were detected using alkaline phosphatase-conjugated goat anti-rabbit IgG with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium as substrates in the presence of 640 mg/liter levamisole (Sigma) to inhibit plant phosphatases. Hand-cut transverse stem and root sections were also stained with phloroglucinol HCl for lignin (C₃–C₆ cinnamaldehydes and C₁–C₆ benzaldehydes) staining.

Phylogenetic Analysis—An *Arabidopsis* P450 data base was constructed using information available at drnelson.utm.edu/BiblioD.html and www.biobase.dk/P450/p450list.shtml. For each family, a consensus sequence was generated using ClustalX Version 1.8 (available at www-igbmc.u-strasbg.fr/BioInfo/) and Genedoc (available at www.psc.edu/biomed/genedoc). Sequences truncated from the hyper-variable membrane anchor up to the proline-rich hinge region were used to generate the alignments and consensus. The final alignment of the consensus sequences and the phylogenetic tree were generated with

ClustalX and Treeview Version 1.5.2 (available at taxonomy.zoology.gla.ac.uk/rod/rod.html).

RESULTS

CYP98A3 Gene Expression in Arabidopsis—The scanning of the numerous CYP98 ESTs available in data banks suggests a significant level of constitutive gene expression in many plant tissues, in particular expression in lignin-rich tissues such as stems, xylem, and fibers. In *Arabidopsis*, CYP98A3 appears as one of the constitutively expressed P450 genes, but ESTs do not give any precise idea of its tissue- or organ-specific expression. RNA blot analysis was thus performed with total RNA from the different plant organs and from leaves lacerated and aged on growth medium to activate genes of phenylpropanoid metabolism involved in repair and defense mechanisms (20) (Fig. 2). This analysis showed that CYP98A3 message was present in all plant tissues, but was by far highest in stems and then in roots and siliques. In leaves, message accumulation was induced by wounding. Expression of CYP98A3 in *Arabidopsis* is thus high in lignin-synthesizing tissues.

Isolation of the CYP98A3 cDNA and Expression of the Protein in Yeast—The coding sequence of CYP98A3, available from genome sequencing, was used to design PCR primers for amplification of the complete cDNA. Restriction sites allowing insertion into the yeast expression vector pYedP60 (14) were added at both ends. The amplicon was first cloned into a pGEM-T vector for complete sequencing before transfer to the expression vector. Galactose-induced expression in the WAT11 yeast strain, coexpressing the *A. thaliana* P450 reductase ATR1, under standard conditions routinely led to the production of \sim 150 pmol of P450/mg of yeast microsomes, *i.e.* 15 nmol/liter of culture (Fig. 3, upper panels). CYP98A8 and CYP98A9, expressed under similar conditions, were usually produced at lower levels. The best preparation contained 107 pmol/mg of microsomal protein and 5.3 nmol/liter of culture for CYP98A8 and 118 pmol/mg of protein and 7 nmol/liter of cul-

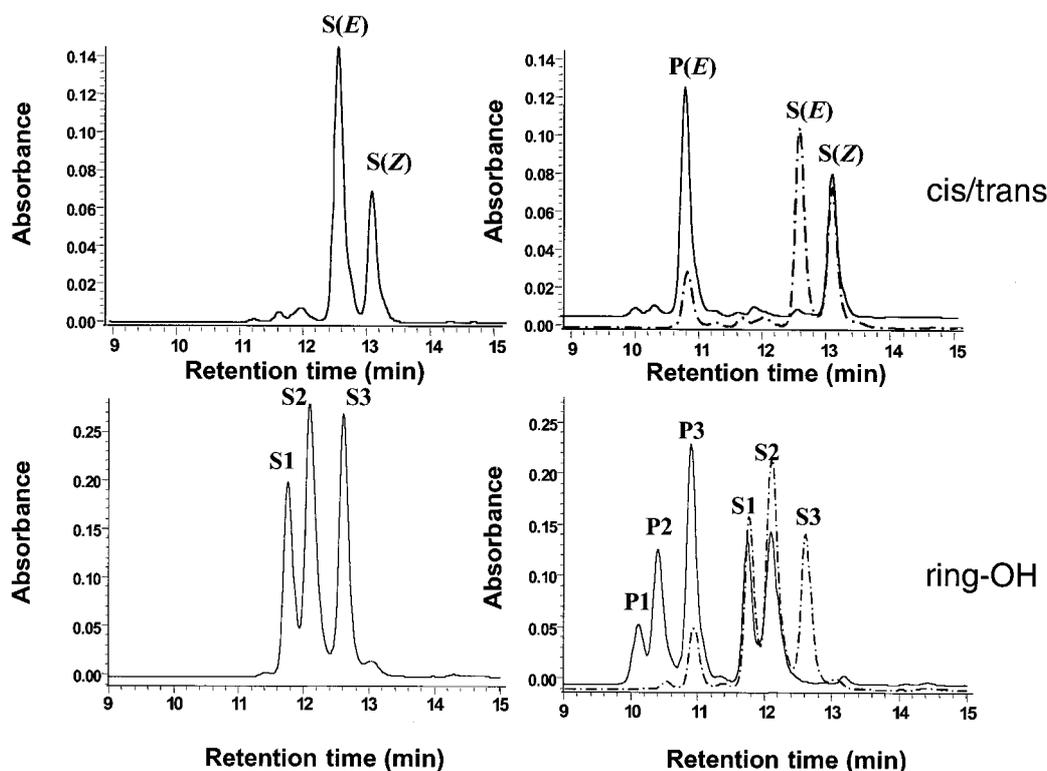


FIG. 6. **Substrate specificity of CYP98A3 for coumaroyl shikimate isomers.** The mix of *cis* and *trans* forms was generated by UV irradiation (upper panels). The mix of 3-, 4-, and 5-isomers was generated by incubation of the 5-isomer for 1 h at 90 °C (lower panel). Left panels, isomers used as substrates or incubated without NADPH. Right panels, metabolites obtained after 5 min (dashed line) or 70 min (solid line) incubation at 28 °C with 1.2 pmol of recombinant CYP98A3. *S(E)* or *S3*, *trans*-5-*O*-(4-coumaroyl)shikimate; *S(Z)*, *cis*-5-*O*-(4-coumaroyl)shikimate; *P(E)* or *P3*, *trans*-5-*O*-(4-caffeoyl)shikimate; *P1*, *trans*-3-*O*-caffeoylshikimate; *P2*, *trans*-4-*O*-caffeoylshikimate; *S1*, *trans*-3-*O*-(4-coumaroyl)shikimate; *S2*, *trans*-4-*O*-(4-coumaroyl)shikimate. Absorbance was monitored at 320 nm. Estimated turnovers are around 600 min⁻¹ for S in both experiments, which shows that isomers are not strong competitors of the metabolism of *trans*-5-*O*-(4-coumaroyl)shikimate.

ture for CYP98A9. Addition of a 4-His tag to the C terminus of CYP98A3 did not significantly alter its level of expression and allowed protein purification for the production of rabbit polyclonal antibodies. These antibodies were specific for CYP98A3 and did not cross-react with yeast-expressed CYP73A1, CYP98A8, or CYP98A9 (Fig. 3, lower panel).

CYP98A3 Is a 3'-Hydroxylase of *p*-Coumaric Acid Esters—Substrate specificity was investigated using recombinant CYP98A3 in yeast microsomes coexpressed with the *A. thaliana* P450 reductase ATR1. Free phenylpropanoids were shown to be the substrates of the P450 enzymes involved in 4- and 5-hydroxylations of the aromatic ring of the C₃–C₆ structure for lignin biosynthesis: cinnamic acid in the case of CYP73 enzymes (8, 21) and coniferylaldehyde, coniferyl alcohol, and, to a lesser extent, ferulic acid in the case of CYP84 enzymes (6, 22). The first tests were thus performed with free *p*-coumarate, *p*-coumaraldehyde, and *p*-coumaroyl alcohol, which did not induce the shift in the CYP98A3 heme iron spin state that would be expected upon binding of a P450 ligand (Type I ligand binding spectrum) (19, 23) or any trace of conversion into a more oxygenated molecule in the presence of NADPH and CYP98A3. Recent data have indicated that the methylation step of caffeic acid into ferulic acid is likely to predominantly occur on a CoA conjugate (17, 24–29). Glucose esters, on the other hand, have been frequently described as alternative free energy-rich precursors of phenolic derivatives (30–32), whereas 4-*O*-β-*D*-glucosides are considered to be detoxification, transport, and storage forms of lignin precursors (33). The CoA ester, 1-*O*-glucoside (glucose ester), and 4-*O*-glucoside of *p*-coumaric acid were thus assayed as substrates of CYP98A3. None of these compounds showed any sign of binding to CYP98A3, and none was converted into a more hydrophilic

product. Two older reports by Heller and Kühnl (7) and Kühnl *et al.* (9) described a P450-catalyzed 3'-hydroxylation of the shikimate and quinate esters of *p*-coumaric acid by microsomal fractions of parsley and carrot cell cultures (Fig. 4). Although the hydroxylation of the quinate ester was obviously linked to the biosynthesis of chlorogenic acid, it was postulated that shikimate esters were just metabolically transient intermediates for the formation of more oxygenated cinnamic acids, including lignin precursors. Competition and inhibition experiments suggested that a single P450 catalyzed both reactions. To investigate further what was so far considered a rather odd hypothesis, we incubated recombinant CYP98A3 with 5-*O*-(4-coumaroyl) *D*-quinic acid or 5-*O*-(4-coumaroyl) shikimate in the presence of NADPH. Both the quinate and shikimate esters were very rapidly converted into a more hydrophilic product (Fig. 5). The reaction was completely dependent on NADPH and CYP98A3; no conversion was obtained upon incubation with microsomes from yeast transformed with a void plasmid. Crude serum from rabbit immunized with purified 4-His-CYP98A3 inhibited the reaction by 50% compared with preimmune serum. Comparison with standards of the HPLC retention times, UV absorption spectra, and negative electrospray mass spectrometry analysis (*m/z* 335.3 for caffeoyl shikimate and *m/z* 353.3 for caffeoyl quinate) of the products indicated the formation of caffeoyl derivatives. This was confirmed by acid hydrolysis of the products, leading to the formation of a product with the characteristics of caffeic acid. As expected from their low sequence homology to CYP98A3, neither CYP98A8 nor CYP98A9 metabolized, even at low rates, the shikimate and quinate esters of *p*-coumaric acid.

Characteristics of the Reactions—Catalytic parameters of the reactions were determined in 0.1 M sodium phosphate buffer

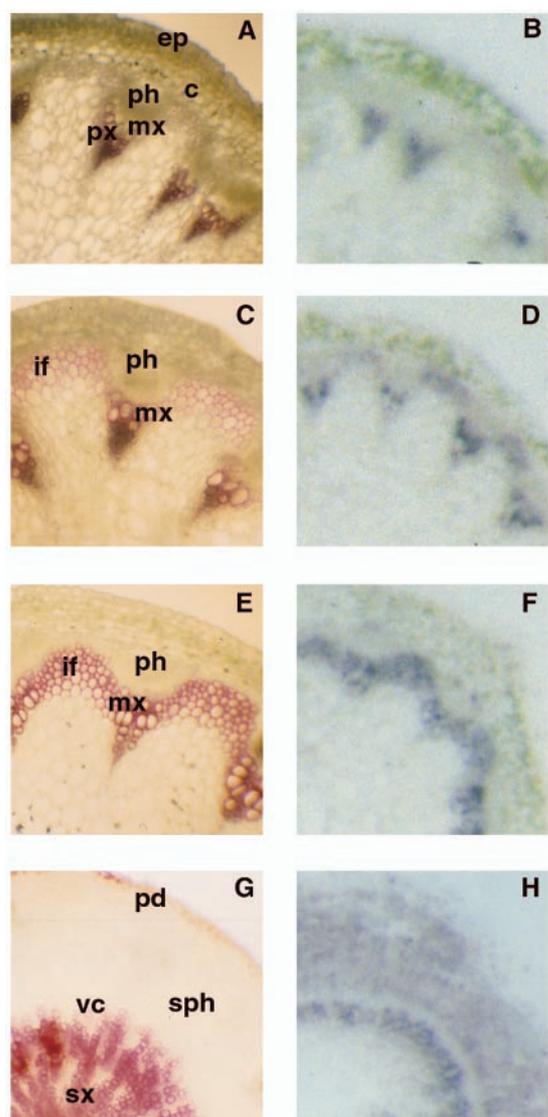


FIG. 7. Immunolocalization of CYP98A3 expression in stems and roots. Hand-cut transversal sections of inflorescence stems and roots were stained with phloroglucinol HCl, a red coloration reflecting lignin content. Adjacent sections were printed onto nitrocellulose and revealed using anti-CYP98A3 polyclonal antibodies. Blue staining is indicative of CYP98A3 expression. In stems, prints were taken at increasing distances from the apical meristem to monitor temporal and developmental expression of CYP98A3 in conjunction with the differentiation of lignified tissues. No blue staining was obtained with pre-immune antibodies. *A, C, E*, and *G*, lignin staining with phloroglucinol; *B, D, F*, and *H*, immunostaining of CYP98A3. *A* and *B*, upper segment of the stem, close to the flower bud; *C* and *D*, mid-stem; *E* and *F*, lower, well differentiated stem close to the rosette; *G* and *H*, root. *ep*, epidermis; *c*, cortex; *px*, protoxylem; *mx*, metaxylem; *ph*, phloem; *if*, interfascicular region; *sx*, secondary xylem; *vc*, vascular cambium; *sph*, secondary phloem; *pd*, periderm.

(pH 7.4) at 28 °C (Table I). Both K_m and K_{cat} favor the metabolism of the shikimate rather than that of the quinate ester, the catalytic efficiency of the enzyme being 4-fold higher with 5-*O*-(4-coumaroyl) shikimate. The K_{cat} of the C3'H is very high compared with those of other plant P450 enzymes expressed in yeast, in particular, higher than what we routinely measured with the recombinant CYP73 enzymes (cinnamate 4-hydroxylases) under similar conditions. A high turnover for the 3'-hydroxylase was predicted by Heller and Kühnl (7) and was already suggested by Ulbricht and Zenk (34).

5-*O*-(4-Coumaroyl) shikimate/quinate easily isomerize from *trans* to *cis* under UV light or from the 5-*O*- to the 3-*O*- and

4-*O*-isomers at physiologic pH (9). The latter process is accelerated at higher temperatures and results from a base-catalyzed intramolecular migration (35). Natural 3-*O*- and 4-*O*-isomers are naturally found in some plant tissues (36–38). As shown in Fig. 6 (upper panels), recombinant CYP98A3 exclusively metabolized the *trans*-isomer of 5-*O*-(4-coumaroyl) shikimate. The *cis*-form remained intact even after complete conversion of the *trans* form. Microsomes from carrot cell cultures were previously reported to exclusively metabolize the 5-*O*-isomer of the quinate ester (9). Recombinant *Arabidopsis* CYP98A3 preferentially hydroxylated the 5-*O*-isomer, but also converted the 4-*O*- and 3-*O*-isomers, although with a lower efficiency (Fig. 6, lower panels). CYP98A3 thus shows a preference for the isomer that is the most abundant under normal conditions and is formed by the *p*-hydroxycinnamoyl-CoA: shikimate *p*-hydroxycinnamoyltransferase, but is also able to cope with other isomers that may arise by isomerization *in planta*, e.g. under heat stress conditions.

Spectrophotometric Detection of Substrate Binding—Our initial screening for potential substrates was performed using spectrophotometric methods for the detection of a shift in the maximum of absorbance of heme that is normally expected upon binding of a substrate (23). This method, which was very useful with other P450 enzymes (19, 40), detected very little change, if any, in P450 absorption upon addition of 5-*O*-(4-coumaroyl) shikimate, despite a high expression of CYP98A3 in yeast microsomes, low affinity, and high rates of metabolism, which imply optimal positioning in the active site. Such an absence of low-to-high spin transition upon substrate binding seems to be shared by other plant P450 enzymes metabolizing compounds with a hydroxyl group next to the position of attack. It possibly means that heme coordination with the hydroxyl oxygen maintains such oxidized P450 enzymes in a low spin state, which would raise some questions concerning their redox potential and interaction with P450 reductases. It may also mean that the active site is naturally devoid of solvent and heme ligand in the resting state.

The CYP98A3 Protein Is Highly Expressed in Lignifying Tissues—The expression pattern of the *CYP98A3* gene and the high turnover of the 3'-hydroxylation reaction favor the hypothesis that the bulk 3-hydroxylation of phenolic compounds occurs on the shikimate or quinate esterified forms of the phenylpropane structure. To test this working hypothesis further, tissue-specific expression of the CYP98A3 protein was visualized in the plant organs showing the highest gene expression. Stem and root transversal sections were printed onto nitrocellulose and revealed with the polyclonal antibodies raised against recombinant CYP98A3 (Fig. 7). Hand sections of neighboring tissues were stained with phloroglucinol HCl to localize lignin accumulation. To follow xylem development in the mature inflorescence stem (41), prints were taken at different distances from the apical meristem. Expression of CYP98A3 correlated with active lignification, as was previously observed for the expression of CCoAOMT in several dicot plants (25, 26). The highest protein expression was detected in differentiating xylem, being first confined in the protoxylem from vascular bundles in the upper part of the stem and then in the metaxylem and interfascicular region, forming a continuous ring in the lower mature stem. In the mature root (Fig. 7, *G* and *F*), some expression was observed in the cortical zone mostly constituted of secondary phloem, but CYP98A3 protein was mainly detected in the ring of differentiating xylem at the periphery of the stele, which is largely formed by lignified secondary xylem (42).

Is 5-*O*-Caffeoyl Shikimate a Substrate of CCoAOMT?—The implication of CYP98A3 in lignification raises the problem of

shed new light on the problem. On the grounds of their phylogeny, high level, and pattern of expression in a broad range of plant species, members of the CYP98 family of P450 enzymes emerged as potential catalysts for the 3-hydroxylation of phenolic compounds. We show here that CYP98A3 from *Arabidopsis* is indeed a 3-hydroxylase of the hydroxycinnamoyl units and that its expression is closely associated with lignification. This P450 does not take *p*-coumaric acid or its CoA ester as a substrate, but both esters of shikimic and quinic acids. The involvement of the shikimate and quinate esters in the biosynthesis of caffeoyl units used for lignification, which is suggested by these data, is in some way a surprise since it introduces a new level of complexity and gridding in the phenylpropanoid pathway. It was, however, very early suggested by the work of Ulbricht and Zenk (34), who demonstrated the existence of a *p*-hydroxycinnamoyl-CoA:shikimate *p*-hydroxycinnamoyltransferase in a broad range of plant species that never accumulate shikimate esters. This work was further supported by the biochemical characterization of a P450-dependent 3'-hydroxylation of the shikimate and quinate esters of *p*-coumaric acid in microsomal fractions of parsley and carrot cell cultures (7, 9) and by the characterization of caffeoyl-CoA 3-*O*-methyltransferase activity in the soluble fraction of the same carrot cell cultures (51). Further investigations in this direction were never pursued, however.

CYP98A3 catalyzes the hydroxylation of two structurally related substrates and their isomers. Such a relaxed substrate specificity is unusual among plant P450 enzymes, with the exception of the enzymes metabolizing fatty acids. It might be needed to compensate the easy interconversion of the isomers. In the case of CYP98A3, the shikimate ester is a better substrate than the quinate ester. This selectivity coincides with that of the *p*-hydroxycinnamoyl-CoA:shikimate *p*-hydroxycinnamoyltransferase previously characterized in radish (52), which also shows a preference for shikimate over quinate for transfer of *p*-coumaroyl from CoA. The more efficient hydroxylation of the quinate ester in carrot, as well as the preference of some transferases for quinate in plants such as potato, *C. endivia*, and apple (37, 52, 53), suggests that C3'H in other plant species might have different substrate preferences or specificities. Differences in substrate preference may also explain accumulation of specific esters in some plant taxa (36). Another remarkable characteristic of C3'H is its high turnover number compared with other P450 oxygenases. Turnover data are not available for the coniferaldehyde 5-hydroxylase (also sometimes called ferulate 5-hydroxylase); but if the yeast-expressed cinnamate 4-hydroxylase was initially reported to have a K_{cat} of 400 min⁻¹ (21), in our hands, it consistently turned over at 50–100 min⁻¹ (19). Cinnamate 4-hydroxylase is usually considered to be a P450 with a very high turnover number, in agreement with its position upstream of a high throughput pathway. The higher turnover (600 min⁻¹ with shikimate) of C3'H, which operates three steps downstream in the same pathway, probably explains why shikimate esters were always described as transient intermediates never accumulating in plant tissues, except for *Palmae*, in which they were considered to be taxonomic markers (37).

A 3-hydroxylation step operating at the level of the shikimate and quinate esters of *p*-coumaric acid opens up many possibilities and raises many questions (Fig. 8). The first is the respective roles of shikimate and quinate esters. If shikimate esters are described as transient intermediates, quinate derivatives such as chlorogenic acid commonly accumulate in some plant species and are alternatively described as growth regulators, disease resistance factors, antioxidants, and compounds affecting organoleptic quality of fruits (36, 38, 54, 55). So, are

the shikimate and quinate esters equivalent in terms of metabolic flux, or does a channeling to the synthesis of lignin and that of accumulated esters exist? Is the quinate ester branch a dead end or a bottleneck? Are other *p*-coumarate esters substrates of C3'H? If a channeling exists, how is it controlled: via 4-coumaroyl-CoA ligases, *p*-hydroxycinnamoyl-CoA *p*-hydroxycinnamoyltransferases, or other enzymes further converting the caffeoyl esters?

This leads to another interesting question concerning the branching of the pathway downstream of C3'H. Our data and previous reports (17, 51) seem to indicate that the shikimate and quinate esters are not substrates of CCoAOMTs. This has to be confirmed in other plant species and with other recombinant CCoAOMTs, but it seems that the caffeate ester(s) have to be converted back to CoA esters for further methylation. Hydroxycinnamoyl-CoA *p*-hydroxycinnamoyltransferases have been described as reversible enzymes (34, 44, 56). Since the exchange between shikimate/quininate and CoA is not energy-consuming, the most likely scenario is that an equilibrium between the ester populations exists in plant cells. The fast and irreversible 3'-hydroxylation would then favor formation of caffeate derivatives and displace pools of conjugates toward more oxygenated structures.

The third question is the connection of the shikimate/quininate ester derivation to other wires of the metabolic grid. One of the possible connections is to the glucose esters, which were reported to be converted to quinate esters via *trans*-esterification (39). If a 3-hydroxylase using a different substrate does not coexist with the *p*-coumaroyl-shikimate/quininate 3'-hydroxylase in higher plants, the channeling of precursors along different wires of the metabolic grids may explain the independent pathways operating for the formation of guayacyl and syringyl precursors needed for lignin biosynthesis (20, 28).

The last and most puzzling question is which kind of evolutionary pressure led plants to use shikimate/quininate esters rather than free acids or CoA or glucose esters for the 3-hydroxylation of hydroxycinnamic acids. Was a P450 metabolizing or binding shikimate derivatives already present and recruited for the reaction? If so, what was the function of this ancestral P450? Or was it the combination of a need to stabilize the very autoxidizable caffeic acid and the extraordinary efficiency of shikimate ester conversion that drove CYP98 evolution? A very attractive hypothesis is that shikimate conjugation was selected since it provides a positive regulation mechanism and optimal tuning of lignin synthesis with the availability of precursors, leaving priority to the synthesis of aromatic amino acids for proteins and other important compounds such as flavonoids.

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