Purification, Cloning, and Properties of an Aeryltransferase Controlling Shikimate and Quinate Ester Intermediates in Phenylpropanoid Metabolism*

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A protein hydrolyzing hydroxycinnamoyl-CoA esters has been purified from tobacco stem extracts by a series of high pressure liquid chromatography steps. The determination of its N-terminal amino acid sequence allowed design of primers permitting the corresponding cDNA to be cloned by PCR. Sequence analysis revealed that the tobacco gene belongs to a plant acyltransferase gene family, the members of which have various functions. The tobacco cDNA expressed in bacterial cells as a recombinant protein fused to glutathione S-transferase. The fusion protein was affinity-purified and cleaved to yield the recombinant enzyme for use in the study of catalytic properties. The enzyme catalyzed the synthesis of shikimate and quinate esters shown recently to be substrates of the cytochrome P450 3-hydroxylase involved in phenylpropanoid biosynthesis. The enzyme has been named hydroxycinnamoyl-CoA:shikimate/quinate hydroxycinnamoyltransferase. We show that p coumaroyl-CoA and caffeoyl-CoA are the best acyl group donors and that the acyl group is transferred more efficiently to shikimate than to quinate. The enzyme also catalyzed the reverse reaction, i.e., the formation of caffeoyl-CoA from chlorogenate (5-O-caffeoyl quinate ester). Thus, hydroxycinnamoyl-CoA:shikimate/quinate hydroxycinnamoyltransferase appears to control the biosynthesis and turnover of major plant phenolic compounds such as lignin and chlorogenic acid.

Phenylpropanoid compounds are derived from phenylalanine by the action of phenylalanine ammonia-lyase, the branch point enzyme between primary and secondary metabolism (see Fig. 1) (1, 2). The biosynthesis of phenylpropanoids is developmentally activated in specific tissues and cell types and also in response to biotic and abiotic stimuli such as wounding, pathogen infection, and ultraviolet irradiation (1, 3–5). Cinnamic acid, the reaction product of phenylalanine ammonia-lyase catalysis, is further modified by the action of hydroxylases and O-methyltransferases, leading to the synthesis of a wide range of hydroxyphenyl acetic acids (see Fig. 1). The enzyme 4-hydroxy-cinnamoyl-CoA ligase catalyzes the formation of CoA esters of hydroxyphenyl acetic acids, which are activated intermediates in the biosynthesis of diverse compounds via specific branches of the pathway leading to lignins, lignans, flavonoids and isoflavonoids, stilbenes, coumarins, and numerous esters and amides (see Fig. 1) (6, 7). 4-Hydroxy-cinnamoyl-CoA ligase from tobacco that uses shikimic acid or quinic acid as acceptor, yielding the shikimate and quinate esters of p-coumarate as substrates, but not the free acid form or the p-coumaroyl-CoA ester (15). Mutants tagged in the p-coumarate 3-hydroxylase gene are characterized by a reduced epidermal fluorescence phenotype (and called ref8) and have been shown to accumulate p-coumarate esters and to be affected in lignin biosynthesis, thus providing direct evidence that p-coumaroyl shikimate and/or p-coumaroyl quinate is probably an important intermediate in the phenylpropanoid pathway (16, 17).

Here, we report the characterization of an acyltransferase from tobacco that uses p-coumaroyl-CoA as acyl donor and shikimic acid or quinic acid as acceptor, yielding the shikimate or quinate ester, respectively. The enzyme has been purified from tobacco stems, and determination of its N-terminal sequence allowed us to clone the cognate cDNA by PCR. The tobacco enzyme shares 46% identity with the carnation anthranilate benzylo-CoA benzoyltransferase, which catalyzes anthranamid phytoalexin biosynthesis (18) and belongs to a large plant acyltransferase gene family (19). The recombinant enzyme expressed in Escherichia coli efficiently synthesizes p-coumaroyl esters from p-coumaroyl-CoA, in agreement with a...
putative role upstream of p-coumarate 3-hydroxylase in the phenylpropanoid pathway. It also catalyzes the biosynthesis of chlorogenic acid (5-O-cafeoylquinic ester), one of the most widespread soluble phenolic compound in the plant kingdom.

The tobacco acyltransferase can also catalyze the reverse reaction, i.e. transfer of the caffeoyl moiety of chlorogenic acid to CoA to form caffeoyl-CoA, the precursor of guaiacyl and syringyl units of lignin. Thus, in plants, hydroxycinnamoyl-CoA:shikimate/quinate hydroxycinnamoyltransferase (HCT\(^1\)) appears to play a critical role in the phenylpropanoid pathway, both upstream and downstream of the 3-hydroxylation step.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Enzymes**

Commonly used chemicals and reagents were of the highest purity readily available. Bradford protein dye reagent was purchased from Bio-Rad (Marnes-la-Coquette, France). Restriction enzymes and buffers were purchased from New England Biolabs, Inc. (Beverly, MA) or Invitrogen (Cergy Pontoise, France). T4 DNA ligase, T4 polynucleotide kinase, ATP, and purified oligonucleotides used for cloning and DNA sequencing were purchased from Invitrogen. Glutathione-agarose, isopropyl-\(\beta\)-thiogalactopyranoside, and LB broth were purchased from Sigma. DNA amplification using Taq polymerase (Invitrogen) was performed in the iCycler\(^{TM}\) thermocycler (Bio-Rad). Plasmid and PCR products were extracted and purified from agarose gels using kits purchased from QIAGEN Inc. (Hilden, Germany).

**Bacterial Strains and Plasmids**

Cloning into the pGEX-KG vector (Amersham Biosciences) and PCR screening for positive clones were carried out as described by Martz et al. (20). Protein expression was performed using \(E.\ coli\) BL21-G612, a kanamycin-resistant strain. \(E.\ coli\) BL21-G612 cells carry the plasmid pLYsS and express rare prokaryotic tRNAs.

**DNA Sequencing**

DNA sequencing was performed using the rhodamine dye terminator cycle ready kit with AmpliTaq DNA polymerase FS (PerkinElmer Life Sciences) and an Applied Biosystems DNA sequencer (Model 373A).

**Partial Purification of HCT from Tobacco Stems and Amino Acid Sequence Analysis**

**Preparation of Crude Extracts**—Tobacco stems (200 g) were ground at 4°C in a Waring Blendor in 200 ml of 20 mM Tris-HCl (pH 7.0) containing 15 mM \(\beta\)-mercaptoethanol (buffer A). The clear supernatant obtained after centrifugation constituted the crude extract.

**Anion Exchange Chromatography**—The crude extract was loaded onto a Mono Q column (0.5 × 5 cm; Amersham Biosciences) equilibrated with buffer A. Proteins were eluted with 200 ml of a 0–0.3 M NaCl gradient in buffer A. Active fractions exhibiting HCT activity were pooled.

**Molecular Exclusion Chromatography under Fast Protein Liquid Chromatography Conditions**—Pooled fractions were concentrated to 200 μl on Centricon 10 concentrators (Amicon, Inc., Beverly, MA), filtered, injected onto a Superdex 75 HR 10/30 column (Amersham Biosciences), and eluted with buffer A at a flow rate of 0.2 ml/min.

**Electrophoresis, Protein Staining, and Microsequencing**—The basic procedures used for electrophoresis under denaturing conditions and silver nitrate staining have been described (21). After electrophoretic separation under denaturing conditions, proteins were immobilized onto Problott membrane (Bio-Rad), stained with Coomassie Blue (Bio-Rad) according to the manufacturer’s instructions, and microsequenced with an Applied Biosystems gas-phase sequencer (Model 470A).

1 The abbreviations used are: HCT, hydroxycinnamoyl-CoA:shikimate/quinate hydroxycinnamoyltransferase; RACE, rapid amplification of cDNA ends; HPLC, high pressure liquid chromatography; GST, glutathione S-transferase.

**Cloning of HCT cDNA**

**Reverse Transcription**—Reverse transcription with total RNA from 2-month-old Nicotiana tabacum stems was carried out using poly(dT) and Superscript\(^{TM}\) (Invitrogen) according to the manufacturer’s instructions.

**Generation of Partial cDNAs**—Partial cDNAs were produced by PCR using cDNA generated by reverse transcription as template. Based on the amino acid sequence of the purified protein, a sense degenerate oligonucleotide primer (SP1) was synthesized (see also Fig. 5): 5′-ATG-GTIAACCCGCGCAGAARCICCC-3′, where I and R indicate inosine and A/G, respectively. The antisense primer (SP1) was based upon a conserved region (DFGWG) near the C terminus of acyltransferase proteins (see Fig. 6) and had the following sequence: 5′-CC CCA RAA RTC-3′. DNA amplification was performed under the following conditions: 94°C for 3 min and then 35 cycles at 94°C for 1 min, 42°C for 1 min, and 72°C for 1 min. At the end of the 35 cycles, the reaction mixture was incubated for an additional 10 min at 72°C. The amplified DNA was resolved by agarose gel electrophoresis, and the band of the expected size (1130 bp) was isolated and subcloned into pCRII-TOPO (Invitrogen) prior to sequencing.

**5′- and 3′-End Amplification**—Based on the nucleotide sequence of the partial cDNA, new oligonucleotide primers were synthesized for amplification of the 5′ and 3′-ends of HCT transcripts (see also Fig. 5): ASP2, 5′-GGGCCAACATTCCTGCAACATCTCAGAAAGC-3′; ASP3, 5′-GAAAATCGAGTGCTGATCGTGACCGACCGAA-3′; ASP4, 5′-GTAATAGAAAGTGTGGATGCTAGTCCAGACCC-3′; and SP2, 5′-AGTTAATCATATGCGATTCCG-3′. To identify the 5′-end of the HCT cDNA, the 5′-RACE System for Rapid Amplification of cDNA Ends Version 2.0 (Invitrogen) was used. First, ASP2 and total RNA from \(N.\ tabacum\) stems were used for cDNA synthesis. Then, a first round of PCR was performed using the nested antisense primer ASP3 along with the anchored RACE-specific primer furnished by the manufacturer. A second round of PCR was performed using 1/10th of the first-round PCR mixture as template and the nested antisense primer ASP4 along with the universal RACE-specific primer (Invitrogen). The amplified DNA was resolved by agarose gel electrophoresis, and the band of the correct size (600 bp) was isolated and subcloned into pCRII-TOPO prior to sequencing. To identify the 5′-end of the HCT transcript, PCR was performed using poly(dT) reverse transcription mixture as template and SP2 and poly(dT) oligonucleotides as primers.

**Generation of Full-length HCT cDNA**—The sequence information required for generation of a full-length cDNA was derived from the nucleotide sequence of the 5′- and 3′-ends of HCT transcripts (see also Fig. 5). The full-length clone was amplified using the end-specific primers (5′-GCTCTAGAGAAGATCGAGGTGAAAGAATCG-3′) and (5′-GCGCCACACATTCCTGCCAACATCTCGTAGGAGC-3′). Amplified PCR products were ligated into the pGEX-KG plasmid for heterologous expression in \(E.\ coli\). DNA amplification was performed under the following conditions: 94°C for 3 min and four cycles at 94°C for 30 s, 46°C for 1 min, and 72°C for 1 min, followed by 30 cycles as above except that the third step was at 55°C for 1 min. After the 30 cycles, the reaction mixture was incubated for an additional 10 min at 72°C. The amplified DNA was resolved by agarose gel electrophoresis, and the band of the correct size (1347 bp) was isolated and subcloned into pCRII-TOPO prior to sequencing.

**Heterologous Expression and Purification of HCT**

The full-length cDNA generated by reverse transcription-PCR was ligated into the pGEX-KG plasmid, which had been digested with the restriction endonucleases XbaI and XhoI. The pGEX-KG plasmid containing the HCT gene coding region were electroporated into \(E.\ coli\) strain BL21-G612. A 10-ml preculture was grown overnight at 37°C in LB medium containing 50 mg/liter kanamycin and 100 mg/liter ampicillin. It was used to inoculate 100 ml of fresh medium. Bacteria were grown for 3 h at 37°C and then transferred to 18°C overnight after harvesting 1 mL isopyrogenic thiogalactopyranoside. After centrifugation for 10 min at 4000 rpm, the bacteria were resuspended in 5 ml of phosphate-buffered saline containing 1% Triton X-100, 2 mM EDTA, 0.1% \(\beta\)-mercaptoethanol, and protease inhibitor mixture tablets (Roche Molecular Biochemicals, Mannheim, Germany). Cells were lysed by two passages through a French press (AmiL) at 11,000 rpm for 30 min; the pellet was discarded; and glutathione-agarose beads (Sigma) were added to the supernatant containing soluble proteins. After 2 h at room temperature, the beads were washed three times with cold phosphate-buffered saline, and the fusion protein was directly cleaved by

\[\text{HCT} \rightarrow \text{HCT cleaved} + \text{Glutathione S-transferase}\]
incubation of the beads with thrombin for 1 h at room temperature. The supernatant contained the recombinant HCT protein. The different steps of purification were assessed by electrophoresis on 12% SDS-polyacrylamide gel. The amount of recombinant HCT protein was quantified by densitometry of the bands on polyacrylamide gels stained with Coomassie Brilliant Blue R-250 (Fluka) and by the method of Bradford (22) using the Bio-Rad reagent.

Synthesis and Purification of Substrates
CoA esters were prepared according to the method of Stockigt and Zenk (27) with some modifications (23) and identified and quantified by spectroscopy as described (24).

Enzyme Activity Measurements
Spectrophotometric Assay—During enzyme purification from plant extracts, 10 nmol of p-coumaroyl-CoA was added to 100 μl of protein mixture and incubated at 30 °C for 1 h. The activity was determined by the decrease in the absorbance at 346 nm measured against a blank reaction mixture containing a boiled protein extract.

Standard Assay Conditions for the Recombinant Enzyme—The reaction mixture contained (in a total volume of 20 μl) 100 mM phosphate buffer (pH 6.6), 1 mM dithiothreitol, 20 ng to 1 μg of purified enzyme, and the different substrates at 10 μM to 10 mM. The reaction was initiated by enzyme addition, incubated at 30 °C for 20 min, and terminated by addition of 20 μl of HPLC solvent. Reaction products were analyzed by HPLC.

Determination of Kinetic Parameters—For K_m determination, varying substrate and enzyme concentrations were used depending on the substrate tested. For K_m with quinate, 50 ng/μl purified enzyme, 1 mM p-coumaroyl-CoA, and 1–10 mM quinate were used. For K_m with shikimate, 1 ng/μl purified enzyme, 1 mM p-coumaroyl-CoA, and 250–4000 μM shikimate were used. For p-coumaroyl-CoA K_m measurement, we used 5 ng/μl purified enzyme with quinate as acceptor and 1.8 ng/μl with shikimate as acceptor, 4 mM quinate or shikimate, and 10–100 μM p-coumaroyl-CoA. For caffeoyl-CoA affinity determination, 20 or 1 ng/μl purified enzyme with quinate or shikimate as acceptor, respectively; 4 mM quinate or shikimate; and 20–200 μM feruloyl-CoA were tested. K_m and V_max values were calculated from the Lineweaver-Burk plots.

Assay Conditions with Other Putative Acyl Donors or Acceptors—Cinnamyl-CoA, sinapyl-CoA, and benzoyl-CoA were tested as acyl donors at a concentration of 100 μM each in the presence of 50 μM purified enzyme and 4 mM quinate or shikimate. Anthranilate, glucose, malate, tyramine, spermidine, spermine, putrescine, and agmatine were tested as possible acyl acceptors at a concentration of 4 mM each in the presence of 50 ng/μl purified enzyme and 4 mM p-coumaroyl-CoA.

Identification of Reaction Products by HPLC
Incubation mixtures were diluted with 1 volume of 0.1% formic acid and 5% acetonitrile in water and resolved on a Waters reverse-phase C18 column (Novapak, 4.6 × 250 mm) using an increasing gradient of acetonitrile (5–50%) in water containing 0.1% formic acid. For the characterization of caffeoyl-CoA formed after incubation of HCT in the presence of chlorogenate and CoA, 20 mM phosphate (pH 5.3) was dissolved in water, and a 5–25% acetonitrile gradient was applied for column elution. Reaction products were characterized by their elution time, and their UV absorption spectra were recorded with a Waters photodiode array detector.

3-O- and 4-O-cafeoylquinic acids were produced from chlorogenic acid (5-O-cafeoylquinic acid; Fluka) by heating for 30 min at 90 °C in 0.2 mM phosphate buffer (pH 7.0) (25). The isomers were separated by HPLC and collected.

Sequence Alignment
Sequence alignment and analysis were performed with ClustalW software. The phylogenetic tree was built using the TreeView program.

RESULTS
Occurrence of an Activity Degrading Caffeoyl-CoA in Tobacco Stems—As shown in Fig. 1, hydroxycinnamoyl-CoA esters are important metabolic intermediates in the phenylpropanoid pathway. We have previously shown that caffeoyl-CoA is methylated by tobacco caffeoyl-CoA O-methyltransferase to yield feruloyl-CoA (10). Surprisingly, when a crude extract from tobacco stems was used as the enzyme preparation, TLC analysis of reaction products revealed the presence of feruloyl-CoA, ferulic acid, and another unknown compound (data not shown). These results suggest that ferulic acid arises from hydrolysis of either feruloyl-CoA or caffeoyl-CoA, followed by the methyla-
tion of caffeic acid, which is known to be catalyzed by caffeic/5-hydroxyferulic acid O-methyltransferase 1 in vitro (10, 26). The reactions involved are indicated by the dotted box in Fig. 1.

To discriminate between these two possibilities, we first investigated the stability of hydroxycinnamoyl-CoA esters in the presence of protein extracts from tobacco stems. As shown in Fig. 2A, the UV absorption spectrum of caffeoyl-CoA presents three maxima of absorption. The peak at 346 nm is characteristic of the presence of the thioester bond (27). After a 1-h incubation of caffeoyl-CoA at 30 °C in the presence of tobacco stem extract (Fig. 2B), the absorbance of the two first peaks remained roughly unchanged, whereas the third absorption peak had markedly decreased. These observations indicate that hydrolysis of caffeoyl-CoA is catalyzed by the crude enzyme extract. No change in the absorption spectrum was recorded in the absence of protein extract (data not shown) or in the presence of a boiled protein extract (Fig. 2A).

**Purification of Tobacco Thioesterase**—The total protein extract from tobacco stems was clarified by centrifugation and filtration. Proteins were first fractionated by anion exchange chromatography under fast protein liquid chromatography conditions (Fig. 3A). Fractions containing thioesterase activity (as measured by the decrease in absorbance at 346 nm) were then pooled, concentrated, and submitted to two successive molecular sieving chromatography steps (Fig. 3, B and C). Table I summarizes the purification factor and yield values measured at each purification step. Likely due to some enzyme instability, a final enzyme activity recovery of 0.6% was observed, resulting in a low apparent purification factor. All our attempts to further purify the enzyme were unsuccessful and led to a complete loss of thioesterase activity. However, when the content of active protein fractions from the second molecular sieving step was analyzed by electrophoresis on SDS gels, only a limited number of protein species were detected (Fig. 3D). Among the protein bands, those in the 45–51-kDa range displayed intensity variations, which correlated with enzyme activity levels. Therefore, fractions 34–40 were pooled, concentrated, and submitted to preparative SDS gel electrophoresis, followed by blotting on membrane. Every protein band in the 45–51-kDa range was microsequenced, and one amino acid sequence revealed important homology to protein sequences available in the data banks: as shown in Fig. 4, the N-terminal amino acid sequence of 18 residues identical to the N terminus of a carnation protein (Fig. 6 presents a few sequences of typical members of the family, the catalytic properties of which have been well characterized. They catalyze the transfer of acetyl, benzoyl, or hydroxycinnamoyl groups onto a variety of acceptor molecules, including alkaloids (salutaridinol 7-O-acetyltransferase and deacetylviandoline 4-O-acetyltransferase), anthranilate (anthranilate N-hydroxycinnamoyl/benzoyltransferase), benzyl alcohol (benzyl-alcohol acetyltransferase), and anthocyanins (anthocyanin 5-aromatic acyltransferase), and diterpenoids (3'-N-debenzoyl-2'-deoxytaxol N-benzoyltransferase). As shown in
Fig. 6. Alignment of N-terminal sequences of tobacco and carnation proteins. Vertical lines indicate the amino acid residues strictly conserved in the two proteins.

Fig. 7. Nucleotide and deduced amino acid sequences of tobacco HCT cDNA. Initiation and termination codons are in boldface. Arrows indicate the positions of sense (SP1–3) and antisense (ASP1–5) primers used for cloning (see "Experimental Procedures").

Fig. 8. Alignment of N-terminal sequences of tobacco and carnation proteins. Vertical lines indicate the amino acid residues strictly conserved in the two proteins.

Substrate Specificity of Tobacco HCT—As mentioned above, the importance of an acyltransferase step in the phenylpropanoid biosynthetic pathway has been strongly suggested by the recent characterization of a cytochrome P450 3-Hydroxy-

TABLE I

Summary of the purification of HCT from tobacco stems

| Purification step | Activity | Protein | Specific activity | Total activity | Yield | Purification factor |
|------------------|----------|---------|------------------|----------------|-------|--------------------|
| Crude extract    | 0.33     | 0.26    | 1.25             | 16.10^3        | 14   | 6                  |
| Ion exchange     | 0.26     | 0.03    | 7.5              | 2.310^3        | 14   | 6                  |
| 1st molecular sieving | 0.15  | 0.015   | 10               | 320            | 2     | 8                  |
| 2nd molecular sieving | 0.085 | 0.017   | 8                | 100            | 0.6   | 6.5                |

Shikimate/Quinate Hydroxycinnamoyltransferase of Tobacco
those of an authentic standard. To quantify the reverse HCT reaction, we also used another solvent system allowing the precise estimation of newly formed caffeoyl-CoA ester (see "Experimental Procedures"). These results indicate that transferase activity may be implicated at different levels in the phenylpropanoid metabolic grid.

To gain insight into HCT function, we evaluated the kinetic parameters of the purified enzyme for a range of substrates. As shown in Table II, the affinity of the enzyme for shikimate as acceptor was 100-fold higher than for quinate. From the data presented in Table II, it also appears that various CoA esters can be used by HCT to transfer the acyl group to shikimate, but with different efficiencies. Caffeoyl-CoA was the most efficient donor, with a $V_{\text{max}}/K_{m}$ of 0.3, followed by $p$-coumaroyl-CoA, which displayed a value of 0.11, whereas feruloyl-CoA was a poor donor, with a 10-fold lower efficiency (Table II). The activity with sinapoyl-CoA was even lower, and the kinetic parameters were not measured with this substrate. When quinate was tested as the acceptor molecule, $p$-coumaroyl-CoA was a better donor compared with caffeoyl-CoA (Table II), whereas no activity at all was recorded with feruloyl-CoA or sinapoyl-CoA (data not shown).

**DISCUSSION**

A thioesterase activity hydrolyzing caffeoyl-CoA was detected in tobacco extracts and purified. From the N-terminal sequence of an authentic standard. To quantify the reverse HCT reaction, we also used another solvent system allowing the precise estimation of newly formed caffeoyl-CoA ester (see "Experimental Procedures"). These results indicate that transferase activity may be implicated at different levels in the phenylpropanoid metabolic grid.

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amino acid sequence of the purified protein, we cloned the corresponding cDNA, which proved homologous to acyltransferase genes from various origins and with diverse functions (Fig. 6). Plant acyltransferases are encoded by a large gene family, the members of which are involved in the biosynthesis of a wide variety of secondary metabolites (18, 19, 32) and display common structural features that enabled us to clone a tobacco homolog. Heterologous expression of the cDNA in *E. coli* yielded a recombinant protein that was purified and characterized. The enzyme was shown to be the most active with *p*-coumaroyl-CoA and caffeoyl-CoA esters as acyl donors, to also use feruloyl-CoA (but less efficiently), and to have very low activity with sinapoyl-CoA. Surprisingly, cinnamoyl-CoA was a good donor (data not shown), but the functionality of the resulting cinnamic esters in the lignin biosynthetic route is not demonstrated because it is not known whether these esters are substrates for cinnamate 4-hydroxylase or *p*-coumarate 3-hydroxylase. It is noteworthy that the tobacco acyltransferase has a pronounced preference for shikimic acid versus quinic acid as acceptor (Table II), but can, however, efficiently catalyze the synthesis of quinate esters (Fig. 8). This contrasts with the strict specificity of acyltransferases partially purified from various plants, which have been reported to transfer the acyl group of CoA esters either to quinate or to shikimate, but not to both (29–31). We have also tested other potential acceptors shown to be active with other plant enzymes, *viz.* anthranilate, glucose, malate, tyramine, spermidine, spermine, putrescine, agmatine, and benzyl alcohol; but no activity could be detected with these compounds (data not shown). Thus, the tobacco enzyme appears to be specialized in the synthesis of quinate and shikimate esters.

**Fig. 8.** HPLC analysis of HCT reaction products. An aliquot of the incubation medium without (left panels) or with (right panels) HCT was analyzed. The nature of the substrates tested is presented in the left panels, and that of the reaction products detected at 320 nm in the right panels. Products were characterized by their retention times, and UV absorbance spectra were recorded with a photodiode array detector.

**Table II**

| Varying substrate | Saturating substrate | Kinetic parameters |  |
|-------------------|----------------------|--------------------|---|
|                   |                      | *Km* (µM)          | *Vmax* (picokatal/s/mg) | *Vmax*/*Km* (picokatal/s/mg·µM) |
| *p*-Coumaroyl-CoA | Shikimate            | 600 ± 150          | 65 ± 20                   | 0.11 |
| Caffeoyl-CoA      | Shikimate            | 50 ± 20            | 15 ± 5                    | 0.3  |
| Feruloyl-CoA      | Shikimate            | 350 ± 100          | 10 ± 5                    | 0.03 |
| *p*-Coumaroyl-CoA | Quinate              | 150 ± 50           | 4.6 ± 1.6                 | 0.03 |
| Caffeoyl-CoA      | Quinate              | 730 ± 800          | 1.4 ± 0.35                | 0.002|
| Shikimate         | *p*-Coumaroyl-CoA    | 750 ± 100          | 140 ± 20                  | 0.19 |
| Quinate           | *p*-Coumaroyl-CoA    | 70.10³ ± 40.10³    | 21 ± 10                   | 0.0003|

**HCT belongs to a versatile plant acyltransferase family that shares structural motifs (Fig. 6) and that comprises several members (the catalytic properties of which have been determined) that are involved in diverse secondary metabolisms of plants. We aligned the sequences of all these biochemically characterized acyltransferases and constructed the phylogenetic tree presented in Fig. 9. It appears that the gene sequences from different plant species cluster within four distinct groups. Groups A and B (Fig. 9) include acyltransferases involved in Taxol and anthocyanidin biosynthesis, respectively. Gene products of group C catalyze the esterification of the hydroxyl moiety of metabolically unrelated molecules, whereas subgroup D comprises HCT and the related enzyme anthranilate *N*-hydroxycinnamoyl/benzoyltransferase, both of which transfer hydroxycinnamoyl groups to acceptors issued from the
FIG. 9. Phylogenetic tree of acyltransferases revealing four evolutionary sequence clusters. The tree was constructed by neighbor-joining distance analysis. Line lengths indicate the relative distances between nodes. Sequences of biochemically characterized enzymes were used for alignment, DBTNBT, 3'-N-debenzylo-3'-deoxytaxol N-benzoyltransferase from T. cuspidata (GenBankTM/EBI accession number AF193765); TAT, taxadienol acetyltransferase from T. cuspidata (accession number AF190130); TBT, taxane benzoyltransferase from T. cuspidata (accession number AF297618); Gs5AT, anthocyanin-5-O-glucoside 6'-O-acyltransferase from G. triflora (accession number BAA74428); Pf5AT, anthocyanin-3-O-glucoside 6'-O-acyltransferase from P. frutescens (accession number BAAB9475); Ss5MaT1, anthocyanin-5-O-glucoside 6'-O-malonyltransferase from S. splendens (accession number AF405267); SaAT, salutaridinol 7-O-acyltransferase from P. somniferum (accession number AF193789); HCT, benzyl-alcohol acetyltransferase from C. breweri (accession number AF339913); PERLAT, deacetylvindoline 4'-O-acetyltransferase from P. frutescens (accession number AF405264); DAT, deacetylvindoline 4-O-acetyltransferase from C. roseus (accession number AF053307); SAT, strawberry alcohol acetyltransferase from F. ananassa (accession number AF193789); SALAT, salutaridinol 7-O-acetyltransferase from P. somniferum (accession number AF339913); BEAT, benzyl-alcohol acetyltransferase from C. breweri (accession number AF043484); AT2G19070 and AT5G57840, A. thaliana genes encoding putative acyltransferases; HCBT, anthranilate N-hydroxycinnamoyltransferase from D. caryophyllus (accession number Z94383); AT5G498930, A. thaliana gene product with activity similar to that of HCT (L. Hoffmann, S. Besseau, P. Geoffroy, and M. Legrand, unpublished data). HCT is from N. tabacum (this work).

The unsuspected importance of p-coumaroylquinic and p-coumaroylshikimic esters in phenylpropanoid metabolism was uncovered by characterization of the Arabidopsis p-coumarate 3-hydroxylase, which very efficiently hydroxylates the two esters, but not p-coumaric acid or p-coumaroyl-CoA (15). In the present work, we cloned and expressed the acyltransferase situated upstream of the 3-hydroxylation step, which furnishes the substrates to the P450 hydroxylase. We have shown that chlorogenic acid (one hydroxylation reaction product), which is the most abundant phenolic compound in tobacco, can yield caffeoyl-CoA when incubated in the presence of CoA and HCT. Thus, HCT plays a dual role in the phenylpropanoid pathway, upstream as well as downstream of the 3-hydroxylation step, as schematically summarized in Fig. 10. Then, caffeoyl-CoA is methylated by caffeoyl-CoA-O-methyltransferase to yield feru-loyl-CoA, the precursor of guaiacyl and syringyl lignins (see Fig. 1). In fact, although free hydroxycinnamic acids have long been thought to be key intermediates in the pathway, it has now been clearly demonstrated that many enzymatic conversions occur instead at the level of hydroxycinnamic esters, aldehydes, and alcohols (33).

The shikimate pathway provides precursors not only of aromatic amino acids, but also of a vast array of secondary metabolites specific to plants (34). Chlorogenic acid (5-O-caffeoylquinic ester) is the most widespread depside in the plant kingdom (7) and is particularly abundant in Asteraceae, Solanaceae, and Rubiaceae (25). In Nicotianae, its biosynthesis has been studied using radiolabeling methods (35) and cell-free preparations of cell suspensions (36). These studies have shown

2 L. Hoffmann, unpublished data.
3 L. Hoffmann, S. Besseau, P. Geoffroy, and M. Legrand, unpublished data.

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that 5-O-cafeoylquinic ester is the precursor of 3- and 4-isomers. Their biosynthesis has been shown to be affected by environmental cues (7, 37), thus pointing to a regulatory role for the acyltransferase step. Moreover, the recently elucidated major role of quinate and shikimate esters as committed intermediates in the biosynthesis of phenylpropanoids demonstrates that quinate and shikimate have a dual role in plant metabolism, both as precursors of aromatic amino acids as stressed above and as acceptors in acyltransferase reactions. Because acyltransferase efficiency is lower with quinate than with shikimate, regulation of transferase activity may depend on the relative importance of the pools of the two acceptors. In this respect, it is interesting to note that the two compounds are directly convertible in the shikimate pathway (34), thus allowing fine-tuned control of their relative amounts. Compared with the considerable amounts of quinate esters found in some plants, it is striking that shikimate ester accumulation has never been reported (31). This may indicate that small quantities of shikimate are sufficient to permit 3-hydroxylation of the aromatic ring at the level of shikimate ester because the subsequent hydrolysis of the ester into caffeoyl-CoA recycles shikimic acid (Fig. 10). No doubt, future in-depth studies of HCT regulation in various physiological situations will uncover a new checkpoint of the phenylpropanoid flux.

New insights into phenylpropanoid pathway have come recently through antisense repression of several enzymes (4, 5, 33, 38, 39). Such data are now needed to fully understand the function of tobacco HCT in planta. Because HCT is likely implicated in the biosynthesis of guaiacyl and syringyl units of lignin, but also in that of caffeylquinic esters, the predominant soluble phenolic compounds in many plants, one can anticipate that HCT activity changes will have an important impact on plant cell metabolism.

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