

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO	
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DEP: 2020 DIRECCION DE NUEVAS CREACIONES	EVE: 378 FASE NACIONAL INCLUIDO CAPITULO I
TRA: 11 PCT-PATENTE DE INVENCION CAPITULOS I Y II	FOLIOS: 6
ACT: 519 PRESENTACION	

DIRECCIÓN DE NUEVAS CREACIONES

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Bogotá, D.C.,

Abogado(a)/Señor(a)
JAIME EDUARDO DELGADO VILLEGAS

Asunto: Radicación: 13-4870- -6
 Trámite: 11
 Evento: 378
 Actuación: 519
 Oficio: 3408
 Folios: 6

Patente de Invención	X			Patente de Modelo de Utilidad	
Vía Nacional					
Vía PCT (Fase Nacional)	X	Capítulo I	X	Capítulo II	
No. Solicitud Internacional	PCT/MY2011/000097				
Fecha de Presentación Internacional	16/06/2011				

Como resultado del Examen de Fondo realizado, con fundamento en el Artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se comunica:

1. Documentos estudiados

Descripción:	Rad. Nº 13-004870-00000-0000	11/Enero/2013
Reivindicaciones:	Rad. Nº 13-004870-00000-0000	30/Enero/2014
Dibujos:	Rad. Nº 13-004870-00000-0000	11/Enero/2013
Prioridad:	MY PI 2010002835	16/Junio/2010

2. Análisis de la Prioridad

Se acepta la reivindicación de Prioridad porque esta solicitud fue presentada dentro del plazo establecido (Art. 9 D 486) y su contenido técnico corresponde con el de la prioridad reivindicada.

3. Objeto de la invención

De acuerdo con lo establecido en el numeral 1.2.2.2 del Capítulo Primero del Título X de la Circular Única para efectos del examen de patentabilidad, se tendrá en cuenta la tasa pagada por el solicitante al momento de comenzar el examen para las reivindicaciones adicionales; esto es que cuando la solicitud contenga más de diez (10)

reivindicaciones y no se haya pagado la tasa complementaria, sólo se estudiarán las cubiertas por la tasa pagada. Por lo tanto, se estudiarán las reivindicaciones 1 a 5.

Título de la solicitud: “Composiciones que constan de ácido shikímico obtenido de materiales a base de palma aceitera y método de producción de las mismas” (Pág. 1, radicado N° 13-004870-00000-0000).

El problema planteado en esta solicitud consiste en la necesidad de un suministrar un método para la producción de ácido shikímico a partir de una fuente renovable.

Para resolver este problema técnico, la solicitud en estudio proporciona un método para la producción de ácido shikímico a partir de extractos de materiales a base de palma aceitera.

El objeto de la presente Solicitud se relaciona con la Clasificación Internacional de Patentes (CIP) C07C 61/00.

4. De la Solicitud de Patente

Reivindicaciones (Art 30 D 486, Art 22 PCT)

La reivindicación 1 define la materia a proteger de una forma muy general, no limitando correctamente el alcance de la invención, ya que no están definidas con claridad las etapas de: *“obtener los extractos de base de aceite de palma”* y *“purificar dicho ácido shikímico a partir de dichos extractos de materiales a base de aceite de palma”*. Se sugiere especificar con exactitud dichas etapas presentándolas como características técnicas en la reivindicación 1 ya que se consideran esenciales para el método.

Las reivindicaciones 1 a 5 no son claras por cuanto mencionan un método sin enseñar la condiciones técnicas del proceso como lo son (temperaturas, presiones, flujos, etc). Se sugiere realizar la aclaración necesaria.

Las reivindicaciones 1 a 5 no son claras porque se usa de forma equitativa la obtención/extracción de compuestos a partir de aceite de palma y la obtención/extracción de compuestos a partir de palma de aceite o palma aceitera. Se sugiere realizar la aclaración necesaria para indicar si se extrae el producto de interés del material basado en residuos de palma de aceite (palma aceitera) o del aceite obtenido del proceso de molienda del raquis de palma (aceite de palma) o los desechos de esa molienda.

5. Determinación del Estado de la Técnica

El estado de la técnica se determinó con base en la fecha de presentación de la solicitud de prioridad invocada, es decir, el 16 de junio de 2010.

Consultadas las fuentes de información con que se cuenta, se encontraron los siguientes documentos que anticipan el objeto de esta solicitud.

Item	Documento	Título	Fecha de Publicación
D1	CO 6480920	“Composición que consta de ácidos cafeoilshikímicos, ácido protocatecoico, hidroxitirosol, ácido hidrobenczoico y sus derivados y método para preparar los mismos”	26/mayo/2009*
D2	Art 1 ¹	“Aminomethylphosphonic Acid Accumulation in Plant Species Treated with Glyphosate”	2008

*fecha de prioridad.

¹Reddy et al. Journal of agricultural and food chemistry. Vol.56. 2008. Págs. 2125 a 2130.

El documento D1² divulga divulga una composición que comprende al menos compuestos principales que incluyen los isómeros del ácido cafeoilshikímico, ácido parahidroxibenzoico, ácido protocatecoico e hidroxitirosol y un método para preparar la misma a partir de palma aceitera incluyendo, pero sin confinarse, el licor vegetal de la molienda de aceite de palma (Págs. 5, 7 y 10).

El documento D2 divulga el aumento del ácido shikímico bajo la inducción del glifosato en plantas tolerantes y no tolerantes a glifosato (Pág. 2128, Col. 2, Párr. 3 a Pág. 2129, Col. 1, Párr. 1, tabla 4).

6. Examen de Patentabilidad (Art 14 D486)

Nivel inventivo (Art 18 D486)

La reivindicación 1 consiste en “*Un método para la producción de ácido shikímico CARACTERIZADO PORQUE comprende las etapas de:...*” (Ver Pág. 7 del Rad. N° 13-004870-00005-0000).

Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica:

Características esenciales	D1	D2
Un método para la producción de ácido shikímico CARACTERIZADO PORQUE comprende las etapas de:	Método para la producción de ácido cafeoilshikímico y otros compuesto fenólicos: (Págs. 5, 7 y 10).	Método donde se determina la presencia de ácido shikímico en plantas (Pág. 2127, Col. 1, Párr. 3).
Obtener extractos a partir de materiales a base de aceite de palma	Se obtienen extractos con compuestos fenólicos a partir de la palma de aceite, licor vegetal de la molienda y efluente de la molienda (Págs. 5, 7 y 10).	No se menciona
por tratamiento de dicho aceite de palma con glifosato;	No se menciona	Se realiza un tratamiento con glifosato a plantas tolerantes y no tolerantes a glifosato (Pág. 2128, Col. 2, Párr. 3 a Pág. 2129, Col. 1, Párr. 1, tabla 4).
Purificar dicho ácido shikímico a partir de dichos extractos de materiales a base de aceite de palma;	Se separa y purifica el ácido cofeoilshikímico de dichos extractos por concentración y cromatografía, entre otras etapas (Págs. 5, 7 y 10). Para la persona versada en la materia el ácido shikímico es precursor de y a su vez derivado del ácido cofeoilshikímico por lo que la presencia de ácido shikímico en plantas que contienen cofeoilshikímico es requerida ³ .	Se separa a partir de extractos el ácido shikímico mediante filtración y cromatografía (Pág. 2127, Col. 1, Párr. 3).
En el que dichos extractos se obtienen de materiales de desecho líquidos de palma aceitera.	Se obtienen extractos con compuestos fenólicos que incluyen al ácido cofeoilshikímico a partir de la palma de aceite, licor vegetal de la molienda y efluente de la molienda (Págs. 5, 7 y 10).	No se menciona

²[http://worldwide.espacenet.com/publicationDetails/originalDocument?](http://worldwide.espacenet.com/publicationDetails/originalDocument?FT=D&date=20101202&DB=EPODOC&locale=en_EP&CC=WO&NR=2010137943A1&KC=A1&ND=4)

FT=D&date=20101202&DB=EPODOC&locale=en_EP&CC=WO&NR=2010137943A1&KC=A1&ND=4

³Hoffmann, et al. Purification, Cloning, and Properties of an Acyltransferase Controlling Shikimate and Quinate Ester Intermediates in Phenylpropanoid Metabolism. The journal of biological chemistry. Vol 278(1). 2003. Pág. 102. Fig. 10.

Los documentos D1 y D2 se consideran el estado de la técnica más cercano a la invención definida en la reivindicación 1, porque divulgan métodos para la extracción de ácido cofeoilshikímico y del ácido shikímico a partir de material vegetal.

La diferencia entre la invención, definida en la reivindicación 1 y el método del documento D1 consiste en que D1 no menciona la extracción del compuesto base que forma el cofeoilshikimato, es decir el ácido shikímico a partir de la palma de aceite tratada con glifosato.

La diferencia entre la invención, definida en la reivindicación 1 y el método del documento D2 consiste en que D2 no menciona la extracción del ácido shikímico tomando como materia prima la palma aceitera.

El efecto que logra el incluir un tratamiento con glifosato a la palma aceitera es que permite producir en esta una mayor concentración de ácido shikímico, para poder obtener así de esa materia prima ácido shikímico de forma más eficiente.

Por lo tanto, el problema técnico objetivo que pretende resolver esta invención se puede formular así “¿cómo modificar el método de D1 para que se lleve a cabo la obtención de ácido shikímico a partir de palma aceitera de forma más eficiente?”.

Sin embargo, el empleo de glifosato para inducir la producción de ácido shikímico en plantas resistentes y no resistentes a glifosato, ya está divulgado en el documento D2 que se refiere al efecto del glifosato en diferentes plantas (Pág. 2128, Col. 2, Párr. 3 a Pág. 2129, Col. 1, Párr. 1, tabla 4).

En consecuencia, una persona normalmente versada en la materia incluiría el glifosato como agente inductor de ácido shikímico en plantas de D2 en el procedimiento de la anterioridad D1, esperando con certeza lograr el método, como la de la reivindicación 1 de la solicitud, lo cual es considerado obvio y, por lo tanto, sin altura inventiva.

Asimismo, el documento D1 divulga las características de las reivindicaciones dependientes así:

Reivindicación 2. Los extractos se obtienen de licor vegetal de aceite de palma (Págs. 5, 7 y 10).

Reivindicación 3. Los extractos son obtenidos de la corriente de licor vegetal de la molienda del aceite de palma (POME) (Págs. 5, 7 y 10).

Reivindicación 4. Los extractos se obtienen de cualquiera material proveniente de la palma aceitera (Págs. 5, 7 y 10).

Reivindicación 5. Los extractos comprenden compuestos fenólicos de aceite de palma (Págs. 5, 7 y 10).

Puesto que las reivindicaciones dependientes 2 a 5 no mencionan características adicionales que hagan inventiva la solicitud, se considera que tales reivindicaciones tampoco tienen nivel inventivo.

7. Conclusión

Los requisitos de Patentabilidad de la presente Solicitud están afectados así:

Item	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	CO 6480920	1 a 5	Nivel inventivo
D2	Art 1	1 a 5	Nivel inventivo

8. Respuesta a los argumentos del señor solicitante

Se aclara que se traslada un nuevo análisis de patentabilidad que afecta el capítulo reivindicatorio por nivel inventivo, en donde se incluye un nuevo documento D2 adicional a la anterioridad D1 previamente mencionada y se reitera que falta especificar con exactitud las etapas de obtención del ácido shikímico como características técnicas en la reivindicación 1 ya que se consideran esenciales para el método.

En referencia al argumento de la solicitante, según el cual el documento D1 no anticipa por novedad el nuevo capítulo reivindicatorio ni sugiere la purificación del ácido shikímico únicamente a partir de extractos de materiales de aceite de palma y de extractos de los efluentes de la molienda de palma de aceite, se encuentra en este análisis de patentabilidad que si bien la novedad se ve superada por la presencia de glifosato en el nuevo método reivindicado, el documento D1 sí enseña, aunque no únicamente, que se pueden obtener extractos a partir de efluentes líquidos y también que se pueden obtener extractos a partir de otros residuos de la palma (Ver Págs. 5, 7 y 10), anticipando así el objeto de la invención por nivel inventivo.

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir del día siguiente de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Se invita al solicitante se sirva indicar, si así lo considera, su renuncia al término faltante de sesenta (60) días para dar respuesta a este requerimiento y presentarla por escrito.

Finalmente, se le recuerda al solicitante que si como respuesta a este requerimiento llegara a modificar el capítulo reivindicatorio y éste contenga más de diez (10) reivindicaciones, no pagará la tasa de modificación voluntaria pero sí la correspondiente al pago por reivindicación adicional.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.



JOSÉ LUIS SALAZAR LÓPEZ
Director de Nuevas Creaciones (c)

El anterior requerimiento fue notificado por fijación en lista, de fecha 2014-03-18

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Aminomethylphosphonic Acid Accumulation in Plant Species Treated with Glyphosate

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Aminomethylphosphonic acid (AMPA) is the most frequently detected metabolite of glyphosate in plants. The objective of this study was to determine if there is any correlation of metabolism of glyphosate to AMPA in different plant species and their natural level of resistance to glyphosate. Greenhouse studies were conducted to determine the glyphosate I_{50} values (rate required to cause a 50% reduction in plant growth) and to quantify AMPA and shikimate concentrations in selected leguminous and nonleguminous species treated with glyphosate at respective I_{50} rates. Coffee senna [*Cassia occidentalis* (L.) Link] was the most sensitive (I_{50} = 75 g/ha) and hemp sesbania [*Sesbania herbacea* (P.Mill.) McVaugh] was the most resistant (I_{50} = 456 g/ha) to glyphosate. Hemp sesbania was 6-fold and Illinois bundleflower [*Desmanthus illinoensis* (Michx.) MacM. ex B.L. Robins. & Fern.] was 4-fold more resistant to glyphosate than coffee senna. Glyphosate was present in all plant species, and its concentration ranged from 0.308 to 38.7 $\mu\text{g/g}$ of tissue. AMPA was present in all leguminous species studied except hemp sesbania. AMPA concentration ranged from 0.119 to 4.77 $\mu\text{g/g}$ of tissue. Shikimate was present in all plant species treated with glyphosate, and levels ranged from 0.053 to 16.5 mg/g of tissue. Non-glyphosate-resistant (non-GR) soybean accumulated much higher shikimate than glyphosate-resistant (GR) soybean. Although some leguminous species were found to be more resistant to glyphosate than others, and there was considerable variation between species in the glyphosate to AMPA levels found, metabolism of glyphosate to AMPA did not appear to be a common factor in explaining natural resistance levels.

KEYWORDS: Aminomethylphosphonic acid; AMPA; glyphosate; glyphosate-resistant crop; shikimic acid; soybean

INTRODUCTION

Glyphosate is a nonselective broad-spectrum herbicide used extensively throughout the world. Glyphosate inhibits the biosynthesis of aromatic amino acids (phenylalanine, tryptophan, and tyrosine), leading to reduced synthesis of proteins and secondary products (1). Furthermore, deregulation of the shikimate pathway leads to general metabolic disruption (2, 3). The molecular target site of glyphosate in the shikimate pathway is 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS). Inhibi-

tion of EPSPS causes greatly increased levels of shikimate accumulation (4, 5).

Glyphosate-resistant (GR) crops, mainly soybean [*Glycine max* (L.) Merr.], cotton (*Gossypium hirsutum* L.), canola (*Brassica napus* L.), and corn (*Zea mays* L.), were created by stable integration of a transgene that codes a glyphosate-insensitive EPSPS (6). Expression of the GR EPSPS helps to maintain normal aromatic amino acid levels in GR crops treated with glyphosate. GR crops are grown in several countries, and their rapid adoption has led to a large increase in the use of glyphosate. Rapid adoption of GR crops is mainly due to simplicity and flexibility of controlling most grasses and broadleaf weeds with a single herbicide at a lower cost.

Glyphosate degrades relatively rapidly in soils by microbial processes (1, 7). The most frequently detected degradation product in soil and water is aminomethylphosphonic acid (AMPA). Little is known about the enzyme(s) involved in the degradation of glyphosate to AMPA in plants. It has been

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conjectured that glyphosate can be metabolized by plants via two pathways similar to those present in microorganisms (1). One involves oxidative cleavage of the C–N bond to yield AMPA and the other breaking of C–P bond by a C–P lyase to generate sarcosine. AMPA is phytotoxic to plant species, although it is considerably less active than glyphosate (1, 8, 9). Although glyphosate is minimally metabolized by plants (2), AMPA is found as a major metabolite in seeds of canola (10), wheat (*Triticum aestivum* L.) (11), field pea (*Pisum sativum* L.), barley (*Hordeum vulgare* L.), flax (*Linum usitatissimum* L.) (12), and GR soybean treated with glyphosate (13). AMPA was also found in foliage of glyphosate-treated crested wheat-grass [*Agropyron cristatum* (L.) Gaertn.] (14). AMPA residues were also detected in leaves and seeds of field-grown GR soybean treated with glyphosate at label use rates, indicating metabolism of glyphosate in GR soybean (15).

Detection of AMPA in leaves, stems, and seeds of several crops including GR soybean following glyphosate application suggests that a plant glyphosate oxidoreductase (GOX) or similar type of enzyme catalyzes this conversion. AMPA is phytotoxic to soybean, although less active than glyphosate; its mode of action is apparently different from that of glyphosate, because GR and glyphosate-sensitive soybeans are equally sensitive to AMPA (9). GOX has been characterized in several genera of bacteria; however, no plant-derived GOX has been described in the literature.

Some leguminous species are more resistant [e.g., hemp sesbania; sicklepod, *Senna obtusifolia* (L.) H.S. Irwin & Barneby] to glyphosate than others [coffee senna; kudzu, *Pueraria montana* var. *lobata* (Willd.) Maesen & S.M. Almeida]. Low levels of resistance to glyphosate in certain leguminous species may be due to differences in GOX activity (rapid detoxification of glyphosate). We have previously reported that AMPA is formed from glyphosate degradation in glyphosate-treated GR soybean (9). In the present study, we investigated if other leguminous species also produce AMPA from glyphosate. The main objective of this study was to determine if differences in glyphosate sensitivity between species can be explained by degradation to AMPA. Because shikimate accumulation is sometimes used as an indicator of sensitivity to glyphosate, a secondary objective was to determine if this factor correlates with interspecies sensitivity to glyphosate. GR and non-GR soybean along with four nonleguminous species were included for comparison.

MATERIALS AND METHODS

General Experimental Conditions. Greenhouse experiments were conducted from July 2006 to March 2007 at USDA-ARS, Southern Weed Science Research Unit, Stoneville, MS. Eleven plant species with varying levels of sensitivity to glyphosate were used in the study (Table 1). The plant species were corn, coffee senna, cowpea [*Vigna unguiculata* (L.) Walpers], hemp sesbania, horseweed [*Conyza canadensis* (L.) Cronq.], Illinois bundleflower, Italian ryegrass [*Lolium perenne* L. ssp. *multiflorum* (Lam.) Husnot], kudzu, sicklepod, soybean, and velvetleaf [*Abutilon theophrasti* Medik.]. Horseweed (from Tunica, MS), coffee senna, hemp sesbania, Illinois bundleflower, and Italian ryegrass (all from Stoneville, MS) seeds were collected from plants growing in the field. Kudzu seed was purchased from Adams-Briscoe Seed Co., Jackson, GA. Cowpea was purchased from Farmers Feed and Supply Co., Leland, MS. Sicklepod and velvetleaf seeds were purchased from Azlin Seed Service, Leland, MS. Corn and soybean was purchased from Jimmy Sanders, Inc., Hollandale, MS. DKC69-72 RR2, a GR variety, and Pioneer 32D99, a non-GR variety, of corn and Asgrow 4603RR, a GR variety, and DP5110S, a non-GR variety, of soybean were used. Plants were grown in 10 cm diameter plastic pots containing a 1:1 (v/v) mixture of soil (Bosket sandy loam, fine-

Table 1. Plant Growth Stage at the Time of Glyphosate Application

plant species ^a	plants used, no./pot	leaves, no./plant	plant height, cm	plant age, weeks
soybean, GR	3	1–2	10–12	3
soybean, non-GR	3	1–2	12–15	3
cowpea	3	1–2	12–15	3
sicklepod	2	3–4	10–12	4
coffee senna	2	3–4	10–12	4
hemp sesbania	2	5–6	13–15	4
Illinois bundleflower	6	4–5	7–10	4
kudzu	5	2–3	5–8	4
velvetleaf	2	5–6	10–12	4
horseweed	1	25–30	10–13 ^b	20
corn, GR	2	1–2	35–40	3
corn, non-GR	2	1–2	35–40	3
Italian ryegrass	6	3–6	10–15	6

^a GR, glyphosate-resistant; non-GR, non-glyphosate-resistant. ^b Horseweed rosette diameter.

loamy, mixed, thermic Mollic Hapludalfs; pH 8.2, 0.5% organic matter, cation exchange capacity = 17 cmol/kg, 51% sand, 37% silt, 12% clay) and potting mix (Jiffy Products of America Inc., Batavia, IL). The greenhouse was maintained at 28/22 °C (±3 °C) day/night temperature with natural light supplemented by sodium vapor lamps to provide a 13 h photoperiod. Plants were subirrigated with water as needed. Plant growth stage and number of plants used for glyphosate treatment are summarized in Table 1. Spray solutions were applied using an indoor spray chamber equipped with an air-pressurized system delivering 190 L/ha at 140 kPa using 8002E flat-fan nozzles.

Glyphosate I₅₀. Glyphosate acid at 0, 0.03, 0.06, 0.12, 0.25, 0.50, 1.00, 2.00, and 4.00 kg/ha was applied to plants. Spray solutions were prepared using technical grade glyphosate acid (>97% purity, Sigma-Aldrich, Allentown, PA) and Tween 20 (Sigma-Aldrich, St. Louis, MO) at 0.5%, v/v. Technical grade glyphosate acid was used to minimize interference from various ingredients in the commercial formulations of glyphosate. Tween 20-treated plants were included as the control. At 14 days after treatment (DAT), plants were excised at the soil surface and shoot fresh weights were recorded. Data are expressed as percent shoot fresh weight reduction as compared to nontreated plants. Treatments were arranged in a randomized complete block design with five or six replications. Data were fitted to a sigmoidal logistic model 1 or 2 to relate percent shoot fresh weight reduction (y) to herbicide rate (x).

$$y = \frac{a}{1 + \exp^{-(x-x_0)b}} \quad (1)$$

In eq 1, *a* is the difference of the upper and lower response limits (asymptotes), *x*₀ is the herbicide rate that results in a 50% reduction in *y* (*I*₅₀), and *b* is the slope of the curve around *x*₀. The regression parameters for eq 1 were computed using SigmaPlot (version 10.0, Systat Software Inc., San Jose, CA).

$$y = c + \frac{d - c}{1 + \exp\{b[\log(x) - \log(e)]\}} \quad (2)$$

In eq 2, the parameter *e* is also denoted *I*₅₀, and it is herbicide rate producing a response halfway between the upper limit, *d*, and lower limit, *c*. The parameter *b* is the relative slope of the curve around *e*. The regression parameters for eq 2 were computed using *R* software (16). The *I*₅₀ rate was determined using eq 1 for non-GR soybean and eq 2 for all other species. Glyphosate levels at 170 g/ha for horseweed (17) and 220 g/ha for Italian ryegrass (18) were used as *I*₅₀ rate.

Glyphosate, AMPA, and Shikimate Accumulation in Glyphosate-Treated Plants. Glyphosate, AMPA, and shikimate levels were quantified following treatment with *I*₅₀ rates of glyphosate. The *I*₅₀ rate for each plant species was selected to avoid plant death and to enable detection of AMPA residues in plants. Glyphosate *I*₅₀ rates of non-GR corn and soybean were used for their respective GR varieties to enable comparison between GR and non-GR types. Preparation of spray solutions and application was as described in the above study. Tween

20-treated plants were included as controls. Plants were harvested at 7 DAT. Plants were clipped at the soil surface, washed with running water, rinsed with distilled water to remove glyphosate remaining on the leaf surface, and blotted dry with paper towels. Treated plant samples consisted of both stem and leaves. All plant samples were air-dried, ground, and analyzed for glyphosate, shikimate, and AMPA. There were five to seven pots per treatment, and treatment was replicated five times. Treatment means with standard deviation are presented.

Extraction of Plant Tissue and Derivatization of Extracts. Plant samples were analyzed for glyphosate, shikimic acid, and AMPA. For glyphosate and AMPA analysis, extraction and derivatization were performed according to published procedures (19), with modifications. One gram of ground tissue (from the combined sample of all plants in each treatment) was extracted with 10 mL of water in a 15 mL centrifuge tube, shaken, placed in a sonicating bath for 20 min, and then centrifuged (Sorvall RC 5C Plus; Kendro Laboratory Products, Asheville, NC) at 47000 rpm, 20 °C, for 20 min. Supernatant was removed. The tissue sample pellet was extracted a second time by adding 10 mL of water, and procedures were performed as in the first extraction. The volume of the combined supernatant was measured, and then 5 μ L of 12.1 M HCl was added and shaken. Four milliliters was transferred to a 20 mL scintillation vial with a Teflon-lined cap, shaken with 4 mL of methylene chloride, and centrifuged (Savant speed vac model SVC 200, Savant Instruments, Inc., Holbrook, NY) for 10 min. A portion (1.8 mL) of the water layer was taken, and 200 μ L of acidic modifier [16 g of KH_2PO_4 , 160 mL of H_2O , 40 mL of methanol (MeOH), 13.4 mL of HCl] was added. One milliliter was loaded to a cation exchange resin column (AG 50W-X8, H^+ ; Bio-Rad Laboratories, Hercules, CA) previously equilibrated with two 5 mL portions of water. The sample was eluted until the level of column bed. CAX mobile phase (160 mL of H_2O , 40 mL of MeOH, 2.7 mL of HCl) (0.7 mL) was added, eluted, and discarded. Twelve milliliters of CAX mobile phase was again added to the column to elute the analytes. The eluate was collected in a 20 mL vial and evaporated to dryness using a Savant speed vac. To the dried sample was added 1.5 mL of CAX mobile phase, and then the vial was placed in a sonicating bath for 30 min. A 20 μ L aliquot was taken and added to 640 μ L of a solution of 2,2,3,3,4,4,4-heptafluoro-1-butanol and trifluoroacetic anhydride (1:2) in a chilled 4 mL vial. The mixture was allowed to equilibrate at room temperature for 10–15 min. The vial was transferred to a heating block at 90 °C for 1 h and then allowed to cool to room temperature. The solvent was evaporated under a stream of nitrogen, and the residue was dissolved in 80 μ L of ethyl acetate containing 0.2% citral; 50 μ L was transferred to a GC vial and analyzed by GC-MS. This method afforded 90 and 86% recoveries of glyphosate and AMPA, respectively, on the basis of duplicate extraction experiments in which samples were fortified with 100 ng standards per gram of sample.

For the analysis of shikimic acid, powdered sample (0.5 g) was placed in an extraction cell with 12.5 mL of deionized water (pH 2, adjusted with HCl), vortexed for 2 min, then extracted using a MARS Xpress Ultra-High Throughput Microwave Digestion System (CEM Corp., Matthews, NC) for 20 s at 400(¼) W. The extract was filtered through a 0.45 μ m filter, and a 100 μ L aliquot was analyzed for shikimic acid by HPLC (Agilent 1200 series HPLC system, Agilent Technologies, Inc., Santa Clara, CA) using a Gemini C18 column, 250 $\times$ 4.6 mm, 5 μ m (Phenomenex, Torrance, CA). The mobile phase consisted of 98% H_2O (pH 3.0) and 2% MeOH in H_2O eluted isocratically, flow rate = 1 mL. Quantification of shikimic acid was performed from a calibration curve of standard shikimic acid (Sigma-Aldrich, St. Louis, MO). The limit of detection (LOD) and limit of quantitation (LOQ) for shikimic acid were 4 and 12.3 ng on column (10 μ L injection), respectively.

GC-MS Analysis of Glyphosate and AMPA in Plant Samples. Analysis of glyphosate and AMPA by GC-MS (Agilent 6890 series GC coupled to a JEOL GCMateII mass spectrometer) was performed using a DB-5 capillary column (J&W Scientific, Inc., Folsom, CA), 30 m length by 0.25 mm i.d. by 0.25 μ m film. The MS detector was a magnetic sector; spectra were acquired in the positive, low resolution, selected ion monitoring mode. The injection port, GC interface, and ionization chamber were maintained at 260, 230, and 120 °C,

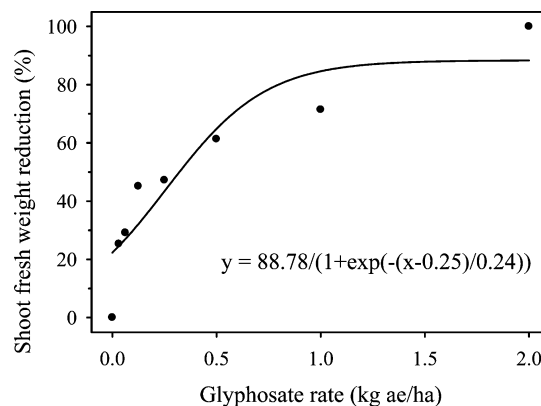


Figure 1. Response of non-glyphosate-resistant soybean to glyphosate at 2 weeks after treatment. Response is expressed as percent shoot fresh weight reduction compared to the nontreated plants. Observed mean values are plotted. Data were fitted to eq 1.

respectively. The carrier gas was ultrahigh-purity helium at a 1 mL/min flow rate. The sample injection volume was 1 μ L. Glyphosate and AMPA in the samples were quantitated from a calibration curve of the respective derivatized standards.

For the analysis of glyphosate and AMPA, the temperature program was as follows: initial, 70 °C, held for 3.5 min, raised to 160 at 30 °C/min rate, raised to 270 at 70 °C/min rate, raised to 310 at 35 °C/min rate, and finally held at this temperature for 3 min. AMPA derivative was observed at 7:23 min (m/z 571, 502, 446, 372) and glyphosate derivative was observed at 7:59 min (m/z 611, 584, 460). The LOD and LOQ for glyphosate were 19.9 and 160 pg on column (1 μ L injection), respectively. The LOD and LOQ for AMPA were 3.71 and 11.2 pg on column (1 μ L injection), respectively. Analysis was performed in duplicate.

RESULTS AND DISCUSSION

Glyphosate I_{50} . Dose–response experiments were conducted to determine glyphosate I_{50} rates, a measure of sensitivity to glyphosate. The observed data and fitted dose–response curves for non-GR soybean are shown in **Figure 1** and for eight other plant species in **Figure 2**. Regression parameters for dose–response curves fitted to eq 2 are presented in **Table 2**. Glyphosate inhibited growth in a nonlinear dose-dependent manner in all plant species. Glyphosate I_{50} rates ranged from 75 to 456 g/ha among the 11 plant species (**Tables 2 and 3**). Coffee senna was most sensitive (I_{50} = 75 g/ha) and hemp sesbania was most resistant (I_{50} = 456 g/ha) to glyphosate. Hemp sesbania was about 6-fold and Illinois bundleflower was about 4-fold more resistant to glyphosate compared to coffee senna. Cowpea, sicklepod, and non-GR soybean were about 3-fold more resistant to glyphosate compared to coffee senna. These results indicate that some leguminous species are more resistant to glyphosate than others. The differential resistance to glyphosate in certain leguminous species may be due to differences in levels of degradation of glyphosate to the much less phytotoxic metabolite of glyphosate, AMPA. To test this hypothesis, we measured glyphosate, shikimate, and AMPA levels in stems and leaves of seven leguminous species including GR soybean following glyphosate treatment at the I_{50} rate. Four nonleguminous species were included for comparison.

Glyphosate, AMPA, and Shikimate Accumulation in Glyphosate-Treated Plants. Glyphosate was present in all plant species 7 DAT, and its concentration ranged from 0.308 to 38.7 μ g/g of tissue (**Table 3**). Because each species was treated using its respective I_{50} rate, comparison of glyphosate concentrations among plant species is not valid. Furthermore, differences in leaf canopy and other biological factors almost certainly

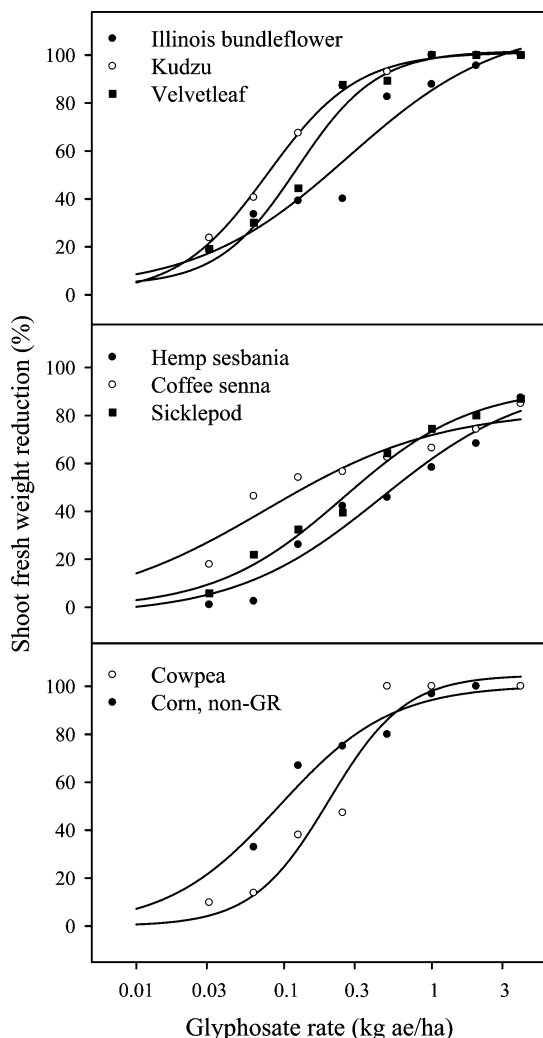


Figure 2. Response of eight plant species to glyphosate at 2 weeks after treatment. Response is expressed as percent shoot fresh weight reduction compared to the nontreated plants. Observed mean values are plotted. Data were fitted to eq 2.

Table 2. Glyphosate I_{50} Rate and Estimated Regression Parameters from Response Curves Fitted to Equation 2^{a,b}

plant species	regression parameters			
	glyphosate I_{50} rate (e), g of ae/ha	slope (b)	lower limit (c)	upper limit (d)
cowpea	201	-1.6780	0.0093	104.75
sicklepod	252	-0.9978	-0.5796	92.07
coffee senna	75	-0.7415	-1.1506	82.57
hemp sesbania	456	-0.8831	-3.1637	94.71
Illinois bundleflower	272	-0.8466	2.1984	113.00
kudzu	77	-1.4331	-0.0006	101.15
velvetleaf	122	-1.6135	3.8723	101.73
corn, non-GR	93	-1.1484	-0.0033	100.50

^a non-GR, non-glyphosate-resistant. ^b I_{50} , glyphosate rate required to cause a 50% reduction in plant growth.

influenced the differences in glyphosate levels between species. Although both GR and non-GR soybeans were treated at the same rate (250 g/ha), GR soybean had a lower glyphosate concentration compared to non-GR soybean. GR soybean possesses insensitive EPSPS enzyme and was not affected by glyphosate. More biomass production in GR soybean after glyphosate treatment resulted in dilution of glyphosate in tissue. Glyphosate translocates to roots, from which it can be exuded

Table 3. Effect of Glyphosate at I_{50} Rate on Glyphosate and Aminomethylphosphonic Acid (AMPA) Concentration at 7 Days after Treatment in Several Plant Species^{a,b}

plant species ^c	glyphosate ^d I_{50} , g of ae/ha	glyphosate, ng/g of tissue	AMPA, ng/g of tissue	ratio, glyphosate/ AMPA
soybean, GR	250	5826 (812)	119 (118)	49
soybean, non-GR	250	25036 (5136)	668 (531)	38
cowpea	201	26763 (5872)	4765 (3589)	6
sicklepod	252	6414 (1985)	1834 (870)	4
coffee senna	75	5906 (4912)	287 (259)	21
hemp sesbania	456	38650 (9022)	nd ^e	
Illinois bundleflower	272	3274 (1471)	1513 (1446)	2
kudzu	77	5561 (2826)	297 (286)	19
velvetleaf	122	678 (153)	nd	
horseweed	170	26326 (5201)	314 (76)	84
corn, GR	93	308 (280)	nd	
corn, non-GR	93	851 (364)	nd	
Italian ryegrass	220	7432 (2724)	nd	

^a I_{50} , glyphosate rate required to cause a 50% reduction in plant growth.

^b Numbers in parentheses indicate one standard deviation. ^c GR, glyphosate-resistant; non-GR, non-glyphosate-resistant. ^d Glyphosate at 250 and 93 g/ha was used for GR soybean and corn, respectively, to enable comparison between GR and non-GR types. Tween 20 at 0.5% (v/v) was added to all treatment solutions.

^e nd, peak not detected or peak below limit of quantitation.

into the soil (20, 21). Differences in this process between GR and non-GR soybean could have also contributed to the differences in glyphosate content. A similar trend was observed between GR and non-GR corn.

AMPA was present in all leguminous species except hemp sesbania (Table 3). AMPA concentration ranged from 119 ng/g of tissue in GR soybean to 4765 ng/g of tissue in cowpea. In another study, 8 μ g of AMPA/g of tissue was detected in GR soybean 7 DAT with glyphosate at 6.72 kg/ha (9). Low levels of AMPA in the present study may be due to the relatively low rate of glyphosate used. Overall, AMPA was detected in six of the seven leguminous species studied. However, there was no correlation between the glyphosate to AMPA ratio (a possible indicator of GOX activity) in the tissue with the glyphosate I_{50} values. Detection of AMPA following glyphosate treatment suggests that a plant GOX may be responsible for this conversion. However, nothing is known about the enzymology of glyphosate degradation to AMPA in plants. AMPA is phytotoxic to both GR and non-GR soybean, and its mode of action is apparently different from that of glyphosate (9). Among four nonleguminous species, AMPA was present in horseweed but not in velvetleaf, Italian ryegrass, and corn (both GR and non-GR).

In nontreated plants, shikimate was present in all plant species and ranged from 2 to 904 μ g/g of tissue (Table 4). Elevated levels of shikimate were observed in all plant species except GR soybean, GR corn, and non-GR corn treated with glyphosate. Shikimate levels ranged from 53 μ g/g of tissue in GR soybean to 16530 μ g/g of tissue in cowpea. Non-GR soybean accumulated much higher shikimate levels than GR soybean. By blocking EPSPS, glyphosate causes many-fold increases in shikimate levels in glyphosate-treated, non-GR soybean (5) and oil seed rape (cv. Iris) (4) plants. In other research, Duke et al. (13) also observed that shikimate levels in GR soybean seed were unaffected by commonly used glyphosate treatments in soybean production. Elevated shikimate levels are used as an early and highly sensitive indicator of glyphosate effects on glyphosate-sensitive plant tissues (4). In transgenic, GR cotton, shikimate levels rise when the plants are treated with enough glyphosate to cause sublethal effects on reproductive tissues

Table 4. Effect of Glyphosate at I_{50} Rate on Shikimate Concentration at 7 Days after Treatment in Several Plant Species^{a,b}

plant species ^c	glyphosate ^d I_{50} , g of ae/ha	shikimate, $\mu\text{g/g}$ of tissue	
		glyphosate treated	nontreated
soybean, GR	250	53 (49)	65 (53)
soybean, non-GR	250	15251 (1132)	27 (21)
cowpea	201	16530 (1809)	64 (36)
sicklepod	252	1092 (164)	348 (313)
coffee senna	75	686 (353)	277 (241)
hemp sesbania	456	6828 (930)	266 (184)
Illinois bundleflower	272	668 (126)	21 (17)
kudzu	77	8915 (368)	188 (109)
velvetleaf	122	159 (213)	2 (2)
horseweed	170	9101 (1614)	251 (234)
corn, GR	93	245 (114)	241 (23)
corn, non-GR	93	149 (48)	161 (72)
Italian ryegrass	220	6701 (920)	904 (231)

^a I_{50} , glyphosate rate required to cause a 50% reduction in plant growth.

^b Numbers in parentheses indicate one standard deviation. ^c GR, glyphosate-resistant; non-GR, non-glyphosate-resistant. ^d Glyphosate at 250 and 93 g/ha was used for GR soybean and corn, respectively, to enable comparison between GR and non-GR types. Tween 20 at 0.5% (v/v) was added to all treatment solutions.

(22). Thus, the absence of an effect on shikimate observed in this study indicated that the insensitive EPSPS was either not inhibited or that the insensitive EPSPS utilized all of the shikimate that would have accumulated from inhibition of the native EPSPS. However, there was no correlation between the absolute amount of accumulated shikimate and the fold increase in shikimate with species sensitivity (I_{50} values) to glyphosate.

These results indicate that AMPA is formed in the plant species treated with glyphosate. However, AMPA data do not support the theory that metabolism of glyphosate explains the relative sensitivities to glyphosate in the species tested. A caveat is that AMPA might be differently metabolized in the different plant species, confounding interpretation of the AMPA data. We know of no papers that have attempted to determine AMPA degradation in plants, although it is reported to degrade in soil by both biotic and abiotic processes (23, 24). Differential natural resistance to glyphosate in leguminous species may have been due to differential interception and retention of spray, absorption, translocation, and sensitivity of EPSPS among the species. Nevertheless, in Illinois bundleflower and, perhaps, sicklepod the ratio of glyphosate to AMPA suggests that metabolic degradation of glyphosate could play a role in the response to glyphosate.

It is also possible that a portion of the AMPA comes from degradation of glyphosate on plant surfaces, followed by uptake of the metabolite. AMPA from soil microbes in contact with root-exuded glyphosate might be translocated to shoots. Recently, an oxidative (abiotic) degradation of glyphosate involving C–P and C–N bond cleavage in the presence of manganese oxide has been suggested as another potential degradation pathway (23). However, the facts that AMPA was not detected in some species and relatively higher levels of AMPA were found in other plant species do not support the view that the AMPA formation is due to conversion of glyphosate to AMPA by leaf surface or root-associated microflora and oxidative degradation. AMPA residues were found in leaves, stems, and seeds of GR soybean following glyphosate applications (9, 13, 15). Furthermore, over time, there is more AMPA in some plant tissues than glyphosate. We found much more AMPA than glyphosate in seeds of glyphosate-treated GR soybean (13). Previous studies have shown axenic cell cultures of soybean to degrade glyphosate much better than cells of other crops (25). Taken together, these data suggest that six of seven

leguminous species may have a plant GOX. However, no plant-derived GOX has been described in the literature, making this an area ripe for further investigation.

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LITERATURE CITED

- (1) Franz, J. E.; Mao, M. K.; Sikorski, J. A. *Glyphosate: A Unique Global Herbicide*; ACS Monograph 189; American Chemical Society: Washington, DC, 1997; 653 pp.
- (2) Duke, S. O. Glyphosate. In *Herbicides: Chemistry, Degradation, and Mode of Action*; Kearney, P. C., Kaufman, D. D., Eds.; Dekker: New York, 1988; Vol. 3, pp 1–70.
- (3) Duke, S. O.; Baerson, S. R.; Rimando, A. M. Herbicides: Glyphosate. In *Encyclopedia of Agrochemicals*; Plimmer, J. R., Gammon, D. W., Ragsdale, N. N., Eds.; Wiley: New York, 2003; <http://www.mrw.interscience.wiley.com/eoa/articles/agr119/frame.html>.
- (4) Harring, T.; Streibig, J. C.; Husted, S. Accumulation of shikimic acid: a technique for screening glyphosate efficiency. *J. Agric. Food Chem.* **1998**, *46*, 4406–4412.
- (5) Lydon, J.; Duke, S. O. Glyphosate induction of elevated levels of hydroxybenzoic acids in higher plants. *J. Agric. Food Chem.* **1988**, *36*, 813–818.
- (6) Padgett, S. R.; Re, D. B.; Barry, G. F.; Eichholtz, D. E.; Delannay, X.; Fuchs, R. L.; Kishore, G. M.; Fraley, R. T. New weed control opportunities: Development of soybeans with a Roundup Ready™ gene. In *Herbicide-Resistant Crops: Agricultural, Environmental, Economic, Regulatory, and Technical Aspects*; Duke, S. O., Ed.; CRC Press: Boca Raton, FL, 1996; pp 53–84.
- (7) Laitinen, P.; Siimes, K.; Eronen, L.; Rämö, S.; Welling, L.; Oinonen, S.; Mattsoff, L.; Ruohonen-Lehto, M. Fate of the herbicides glyphosate, glufosinate-ammonium, phenmedipham, ethofumesate and metamitron in two Finnish arable soils. *Pest Manag. Sci.* **2006**, *62*, 473–491.
- (8) Hoagland, R. E. Effects of glyphosate on metabolism of phenolic compounds: VI. Effects of glyphosine and glyphosate metabolites on phenylalanine ammonia-lyase activity, growth, and protein, chlorophyll, and anthocyanin levels in soybean (*Glycine max*) seedlings. *Weed Sci.* **1980**, *28*, 393–400.
- (9) Reddy, K. N.; Rimando, A. M.; Duke, S. O. Aminomethylphosphonic acid, a metabolite of glyphosate, causes injury in glyphosate-treated, glyphosate-resistant soybean. *J. Agric. Food Chem.* **2004**, *52*, 5139–5143.
- (10) Cessna, A. J.; Darwent, A. L.; Townley-Smith, L.; Harker, K. N.; Kirkland, K. J. Residues of glyphosate and its metabolite AMPA in canola seed following preharvest applications. *Can. J. Plant Sci.* **2000**, *80*, 425–431.
- (11) Cessna, A. J.; Darwent, A. L.; Kirkland, K. J.; Townley-Smith, L.; Harker, K. N.; Lefkovich, L. P. Residues of glyphosate and its metabolite AMPA in wheat seed and foliage following preharvest applications. *Can. J. Plant Sci.* **1994**, *74*, 653–661.
- (12) Cessna, A. J.; Darwent, A. L.; Townley-Smith, L.; Harker, K. N.; Kirkland, K. J. Residues of glyphosate and its metabolite AMPA in field pea, barley, and flax seed following preharvest applications. *Can. J. Plant Sci.* **2002**, *82*, 485–489.
- (13) Duke, S. O.; Rimando, A. M.; Pace, P. F.; Reddy, K. N.; Smeda, R. J. Isoflavone, glyphosate, and aminomethylphosphonic acid levels in seeds of glyphosate-treated, glyphosate-resistant soybean. *J. Agric. Food Chem.* **2003**, *51*, 340–344.
- (14) Cessna, A. J.; Waddington, J. Dissipation of glyphosate and its metabolite AMPA in established crested wheatgrass following spring application. *Can. J. Plant Sci.* **1995**, *75*, 759–762.
- (15) Arregui, M. C.; Lenardón, A.; Sanchez, D.; Maitre, M. I.; Scotta, R.; Enrique, S. Monitoring glyphosate residues in transgenic glyphosate-resistant soybean. *Pest Manag. Sci.* **2003**, *60*, 163–166.

- (16) Ritz, C.; Streibig, J. C. Bioassay Analysis using R. *J. Statist. Software* **2005**, *12*, 1–22; <http://www.bioassay.dk>, accessed Sept 5, 2007.
- (17) Koger, C. H.; Poston, D. H.; Hays, R. M.; Montgomery, R. F. Glyphosate-resistant horseweed (*Conyza canadensis*) in Mississippi. *Weed Technol.* **2004**, *18*, 820–825.
- (18) Nandula, V. K.; Poston, D. H.; Eubank, T. W.; Koger, C. H.; Reddy, K. N. Differential response to glyphosate in Italian ryegrass (*Lolium multiflorum*) populations from Mississippi. *Weed Technol.* **2007**, *21*, 477–482.
- (19) Alferness, P. L.; Wiebe, L. A. Determination of glyphosate and aminomethylphosphonic acid in crops by capillary gas chromatography with mass-selective detection: collaborative study. *J. AOAC Int.* **2001**, *84*, 823–846.
- (20) Coupland, D.; Caseley, J. C. Presence of ^{14}C activity in root exudates and guttation fluid from *Agropyron repens* treated with ^{14}C labeled glyphosate. *New Phytol.* **1979**, *83*, 17–22.
- (21) Kremer, R. J.; Means, N. E.; Kim, S. Glyphosate affects soybean root exudation and rhizosphere microorganisms. *Int. J. Environ. Anal. Chem.* **2005**, *85*, 1165–1174.
- (22) Pline, W. A.; Wilcut, J. W.; Duke, S. O.; Edmisten, K. L.; Wells, R. Accumulation of shikimic acid in response to glyphosate applications in glyphosate-resistant and conventional cotton (*Gossypium hirsutum* L.). *J. Agric. Food Chem.* **2002**, *50*, 506–512.
- (23) Barrett, K. A.; McBride, M. B. Oxidative degradation of glyphosate and aminomethylphosphonic acid by manganese oxide. *Environ. Sci. Technol.* **2005**, *39*, 9223–9228.
- (24) Forlani, G.; Mangiagalli, A.; Nielsen, E.; Suardi, C. M. Degradation of the phosphonate herbicide glyphosate in soil: evidence for a possible involvement of unculturable microorganisms. *Soil Biol. Biochem.* **1999**, *31*, 991–997.
- (25) Komossa, D.; Gennity, I.; Sandermann, H. Plant metabolism of herbicides with C-P bonds: glyphosate. *Pestic. Biochem. Physiol.* **1992**, *43*, 85–94.

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**ENZYME CATALYSIS AND
REGULATION:**

**Purification, Cloning, and Properties of an
Acyltransferase Controlling Shikimate and
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Purification, Cloning, and Properties of an Acyltransferase 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 was 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/quinat 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/quinat 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 hydroxycinnamic acids (see Fig. 1). The enzyme 4-hydroxycinnamoyl-CoA ligase catalyzes the formation of CoA esters of hydroxycinnamic 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-Hydroxycinnamoyl-CoA ligase from various plant species accepts *p*-coumarate, ferulate, and caffeate as substrates in this decreasing preferential order, but does not accept sinapate (8, 9). Thus, it is likely that sinapoyl-CoA is formed via the alternative pathway involving caffeoyl-CoA *O*-methyltransferase, which produces feruloyl-CoA (10, 11), which is the substrate of cinnamoyl-CoA reductase, the first committed enzyme of monolignol synthesis (see Fig. 1). Recently, it has been shown that coniferaldehyde, the product resulting from cinnamoyl-CoA reductase action, is a preferential substrate for the cytochrome P450 hydroxylase that introduces the hydroxyl group in the 5-position of the aromatic ring (see Fig. 1) (12, 13). Finally, the second methylation reaction leading to syringyl lignin subunits may occur at the level of CoA ester, aldehyde, or alcohol derivatives (see Fig. 1) (10, 13, 14).

Very recently, it has been demonstrated that C-3 hydroxylation does not take place at the free acid level, as is the case for C-4 hydroxylation. By a functional genomics approach, *p*-coumarate 3-hydroxylase from *Arabidopsis thaliana* has been shown to be a P450 enzyme that accepts 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 benzoyl-CoA benzoyltransferase, which catalyzes anthranamide 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

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AJ507825.

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putative role upstream of *p*-coumarate 3-hydroxylase in the phenylpropanoid pathway. It also catalyzes the biosynthesis of chlorogenic acid (5-*O*-caffeoylquinic 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- β -D-thiogalactopyranoside, and LB broth were purchased from Sigma. DNA amplification using *Taq* polymerase (Invitrogen) was performed in the iCyclerTM 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 β -mercaptoethanol and 0.2 g of charcoal (Merck). After filtration through four layers of cheesecloth, the homogenate was centrifuged at 10,000 $\times g$ for 30 min. The supernatant was desalted on a Sephadex G-25 column (4 $\times$ 70 cm; Amersham Biosciences) equilibrated with 20 mM Tris-HCl (pH 7.0) containing 15 mM β -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 $\times$ 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 μ l on Centricon 10 concentrators (Amicon, Inc., Beverly, MA), filtered, injected onto a Superdex 75 HR10/30 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) primer and SuperscriptTM (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-GTIAARCCIGCIACIGARACICC-3', where I and R indicate inosine and A/G, respectively. The antisense primer (ASP1) 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 ICC 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'-GCGCCACACATTCCTGCCAACATCTCGTAGGAGC-3'; ASP3, 5'-GAAACTGAGGCTGAGGTGGATCAGCAGCAGCGA-3'; ASP4, 5'-GTCAATGAAAGGTGGGTGGGTGAGGTCCAGACC-3'; and SP2, 5'-AGTAAATTACATGATGCATTGGC-3'. To identify the 5'-end of the HCT transcript, 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 1/100th 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 3'-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 SP3 (5'-GCTCTAGACATGAAGATCGAGGTGAAAGAATCG-3') and ASP5 (5'-CCGCTCGAGTCGACCATGGTCAAAAGTCATACAAG-AACTTCTC-3'). SP3 and ASP5 contain recognition sites for the restriction endonucleases *Xba*I and *Xho*I, which permit subcloning 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 *Xba*I and *Xho*I. The pGEX-KG plasmids 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 addition of 1 mM isopropyl- β -D-thiogalactopyranoside to induce protein expression. 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% β -mercaptoethanol, and protease inhibitor mixture tablets (Roche Molecular Biochemicals, Mannheim, Germany). Cells were lysed by two passages through a French press (Aminco). The bacterial lysate was centrifuged 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

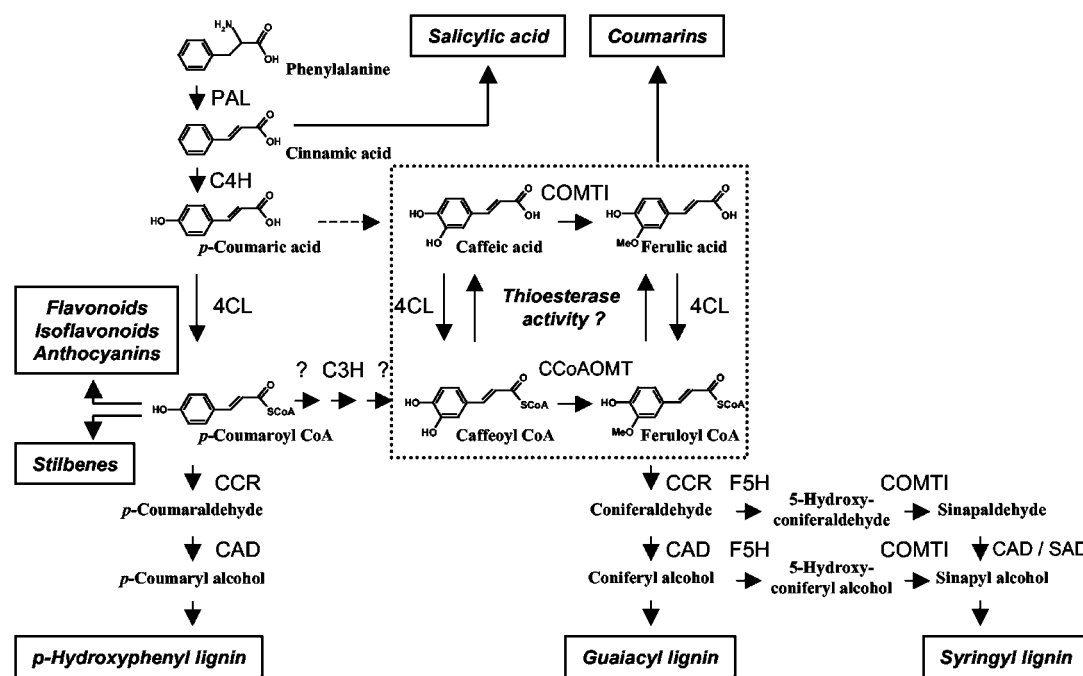


FIG. 1. **Outline of the phenylpropanoid biosynthetic pathway.** Routes to major products are indicated. The dotted box includes the reactions that may explain the appearance of ferulic acid after caffeoyl-CoA incubation with tobacco extract (see "Results"). 4CL, 4-hydroxycinnamoyl-CoA ligase; C3H, *p*-coumarate 3-hydroxylase; C4H, cinnamate 4-hydroxylase; CAD, cinnamyl-alcohol dehydrogenase; CCoAOMT, caffeoyl-CoA *O*-methyltransferase; CCR, cinnamoyl-CoA reductase; COMTI, caffeic/5-hydroxyferulic acid *O*-methyltransferase I; F5H, ferulate 5-hydroxylase; PAL, phenylalanine ammonia-lyase; SAD, sinapyl-alcohol dehydrogenase.

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 Stöckigt 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 μ l 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 reagent.

Standard Assay Conditions for the Recombinant Enzyme—The reaction mixture contained (in a total volume of 20 μ l) 100 mM phosphate buffer (pH 6.6), 1 mM dithiothreitol, 20 ng to 1 μ g of purified enzyme, and the different substrates at 10 μ M to 10 mM. The reaction was initiated by enzyme addition, incubated at 30 °C for 20 min, and terminated by addition of 20 μ l 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/ μ l purified enzyme, 1 mM *p*-coumaroyl-CoA, and 1–10 mM quinate were used. For K_m with shikimate, 1 ng/ μ l purified enzyme, 1 mM *p*-coumaroyl-CoA, and 250–4000 μ M shikimate were used. For *p*-coumaroyl-CoA K_m measurement, we used 5 ng/ μ l purified enzyme with quinate as acceptor and 1.8 ng/ μ l with shikimate as acceptor, 4 mM quinate or shikimate, and 10–100 μ M *p*-coumaroyl-CoA. For caffeoyl-CoA affinity determination, 20 or 1 ng/ μ l purified enzyme with quinate or shikimate as acceptor, respectively; 4 mM quinate or shikimate; and 20–500 μ M caffeoyl-CoA were tested. Finally, for feruloyl-CoA K_m measurement, 8 ng/ μ l purified enzyme, 4 mM shikimate, and 20–200 μ M feruloyl-CoA were used. K_m and V_{max} values were calculated from the Lineweaver-Burk plots.

Assay Conditions with Other Putative Acyl Donors or Acceptors—Cinnamoyl-CoA, sinapoyl-CoA, and benzoyl-CoA were tested as acyl donors at a concentration of 100 μ M each in the presence of 50 ng/ μ l

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/ μ l purified enzyme and 4 mM *p*-coumaroyl-CoA.

Assay for Caffeoyl-CoA Synthesis from Chlorogenate—10 ng/ μ l purified enzyme was incubated in the presence of 100 μ M chlorogenate and 100 μ M 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 μ m, 4.6 $\times$ 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*-caffeoylquinic acids were produced from chlorogenic acid (5-*O*-caffeoylquinic acid; Fluka) by heating for 30 min at 90 °C in 0.2 M 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-

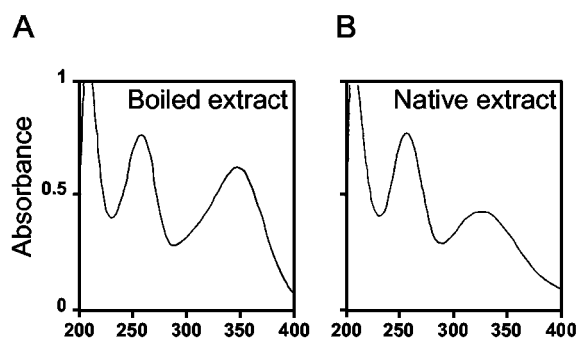


FIG. 2. UV absorbance spectra of caffeoyl-CoA. Absorbance was recorded from 200 to 400 nm after incubation in the presence of boiled (A) or native (B) tobacco stem protein extract.

tion of caffeic acid, which is known to be catalyzed by caffeic/5-hydroxyferulic acid *O*-methyltransferase I *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 a 51-kDa tobacco protein (Fig. 3D, arrow) displayed 12 of 18 residues identical to the N terminus of a carnation protein that has been characterized as a hydroxycinnamoyl/benzoyl-CoA:anthranilate *N*-hydroxycinnamoyl/benzoyltransferase involved in the biosynthesis of dianthramides (18).

Cloning and Purification of the Recombinant Protein—The N-terminal sequence of the HCT protein purified from tobacco and the consensus sequences found in the acyltransferase family (19) were used to design primers for the first step of PCR

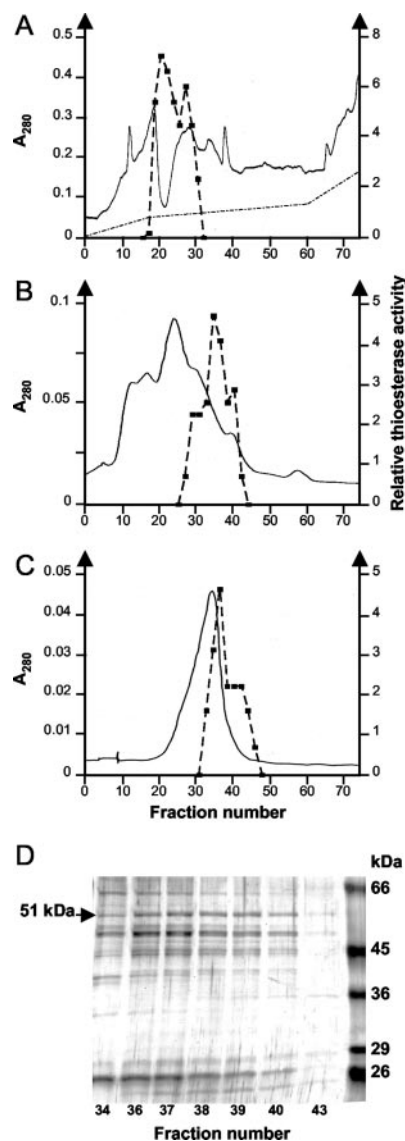


FIG. 3. Purification of tobacco thioesterase by HPLC. Protein elution profiles (solid lines) and thioesterase activity profiles (dashed lines) after ion exchange chromatography (A) and two successive molecular sieving steps (B and C) are shown. Active fractions from the separation shown in C were analyzed by silver staining (D). In D, the arrow indicates the position of the 51-kDa protein that proved to be HCT; protein markers of the given molecular masses appear on the right.

cloning (see "Experimental Procedures"). At the end of the cloning procedure, a nearly full-length tobacco cDNA clone was obtained, the sequence of which is shown in Fig. 5. The closest match found in the data banks (considering only characterized gene products) was still with the anthranilate *N*-hydroxycinnamoyl/benzoyl transferase from carnation, which shares 46% identity and 62% similarity with the tobacco protein. Both proteins belong to a multifunctional superfamily of plant acyltransferases (19). 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 deacetylvindoline 4-*O*-acetyltransferase), anthranilate (anthranilate *N*-hydroxycinnamoyl/benzoyltransferase), benzyl alcohol (benzyl-alcohol acetyltransferase), anthocyanins (anthocyanin 5-aromatic acyltransferase), and diterpenoids (3'-*N*-debenzoyl-2'-deoxytaxol *N*-benzoyltransferase). As shown in

TABLE I
Summary of the purification of HCT from tobacco stems

Enzyme activity was measured by the decrease in absorbance at 346 nm ($\Delta A_{346 \text{ nm}}$), and protein amount was estimated by the Bradford method (22) as described under "Experimental Procedures."

Purification step	Activity	Protein	Specific activity	Total activity	Yield	Purification factor
	$\Delta A_{346 \text{ nm}}/\text{h}/\mu\text{l}$	$\mu\text{g}/\mu\text{l}$	$\Delta A_{346 \text{ nm}}/\text{h}/\mu\text{g}$	$\Delta A_{346 \text{ nm}}/\text{h}$	%	fold
Crude extract	0.33	0.26	1.25	16.10 ³		
Ion exchange	0.26	0.03	7.5	2.3.10 ³	14	6
1st molecular sieving	0.15	0.015	10	320	2	8
2nd molecular sieving	0.085	0.01	8	100	0.6	6.5



FIG. 4. Alignment of N-terminal sequences of tobacco and carnation proteins. Vertical lines indicate the amino acid residues strictly conserved in the two proteins.

Fig. 6, all these proteins have structural features in common, in particular an HXXXD motif (His¹⁵³–Asp¹⁵⁷) that may function in acyl transfer catalysis and the DFGWG block (Asp³⁸²–Gly³⁸⁶) that was used to design cloning primers (see "Experimental Procedures"). To investigate the potential catalytic activity of the tobacco protein, the cloned cDNA was transformed into *E. coli* bacterial cells, and expression of a GST fusion protein was induced as described under "Experimental Procedures." The fusion protein, present in the soluble fraction of transformed bacteria (Fig. 7, lane 6), was purified by affinity chromatography on glutathione-agarose beads and then cleaved by thrombin, yielding the recombinant protein as illustrated in lane 8. The purified recombinant enzyme was used for substrate specificity studies.

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'-hydroxylase that displays a high level of specificity for depsides *versus* free coumaric acid (15). Because *p*-coumaroyl quinate and *p*-coumaroyl shikimate were shown to be substrates for the P450 hydroxylase, we investigated the possibility that the tobacco enzyme catalyzes the synthesis of these compounds. Thus, we incubated the purified recombinant enzyme in the presence of *p*-coumaroyl-CoA and quinic acid or shikimic acid and analyzed the reaction products by HPLC (Fig. 8, A and B). HPLC profiles recorded after incubation of substrates with (*right panels*) or without (*left panels*) active enzyme preparations are shown. In the presence of active enzyme, a new product was detected in both cases, the absorbance spectrum of which was similar to that of *p*-coumaric acid due to the presence of the *p*-coumaroyl moiety (data not shown). The reaction products were further identified by comparing their retention times with those of authentic quinate and shikimate esters of *p*-coumaric acid (15). *In planta*, these compounds are converted by the P450 hydroxylase into the corresponding caffeoyl esters, which, in turn, must be transformed into caffeoyl-CoA ester, which is the substrate of caffeoyl-CoA *O*-methyltransferase, the next enzyme in the pathway (Fig. 1). Because plant acyltransferases have been described as having reversible activity (28–31), we investigated whether the tobacco enzyme can also catalyze the reverse reaction, *i.e.* the formation of caffeoyl-CoA from quinate ester. When chlorogenic acid (5-*O*-caffeoylquinic ester) was incubated in the presence of HCT and CoA (Fig. 8C), it decreased in quantity (reflected by diminished peak height), whereas a small peak of caffeoyl-CoA appeared. The caffeoyl-CoA peak was identified by comparing the elution time and spectroscopic properties with

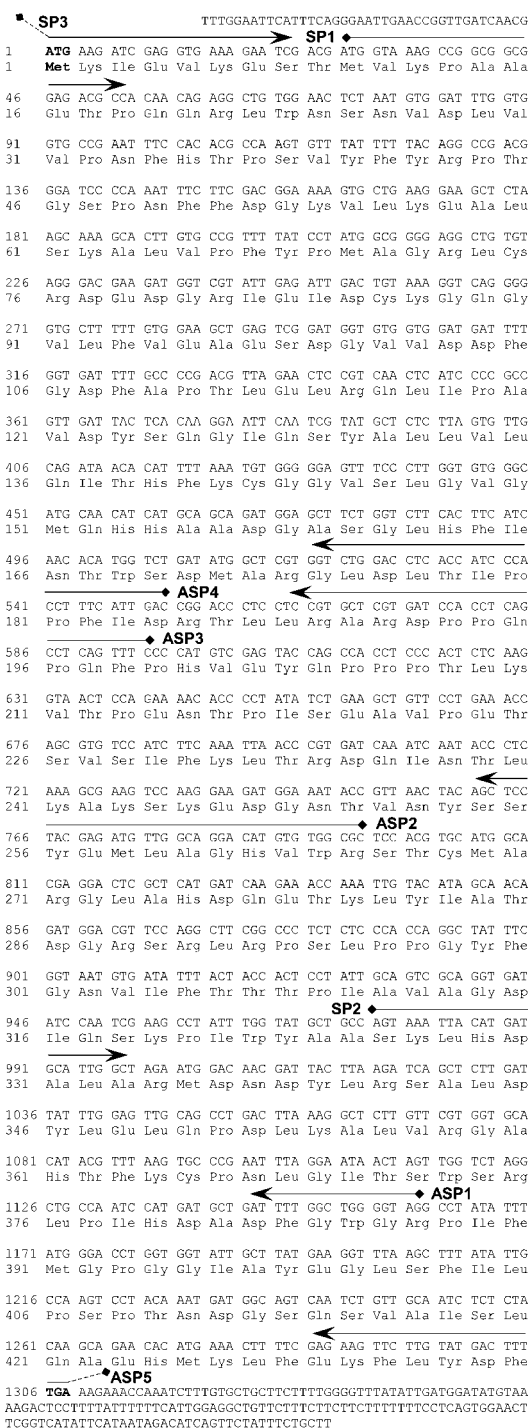


FIG. 5. Nucleotide and deduced amino acid sequences of tobacco HCT cDNA. Initiation and termination codons are in boldface. Arrows indicate the positions of sense (SP1–3) and antisense (ASP1–5) primers used for cloning (see "Experimental Procedures").

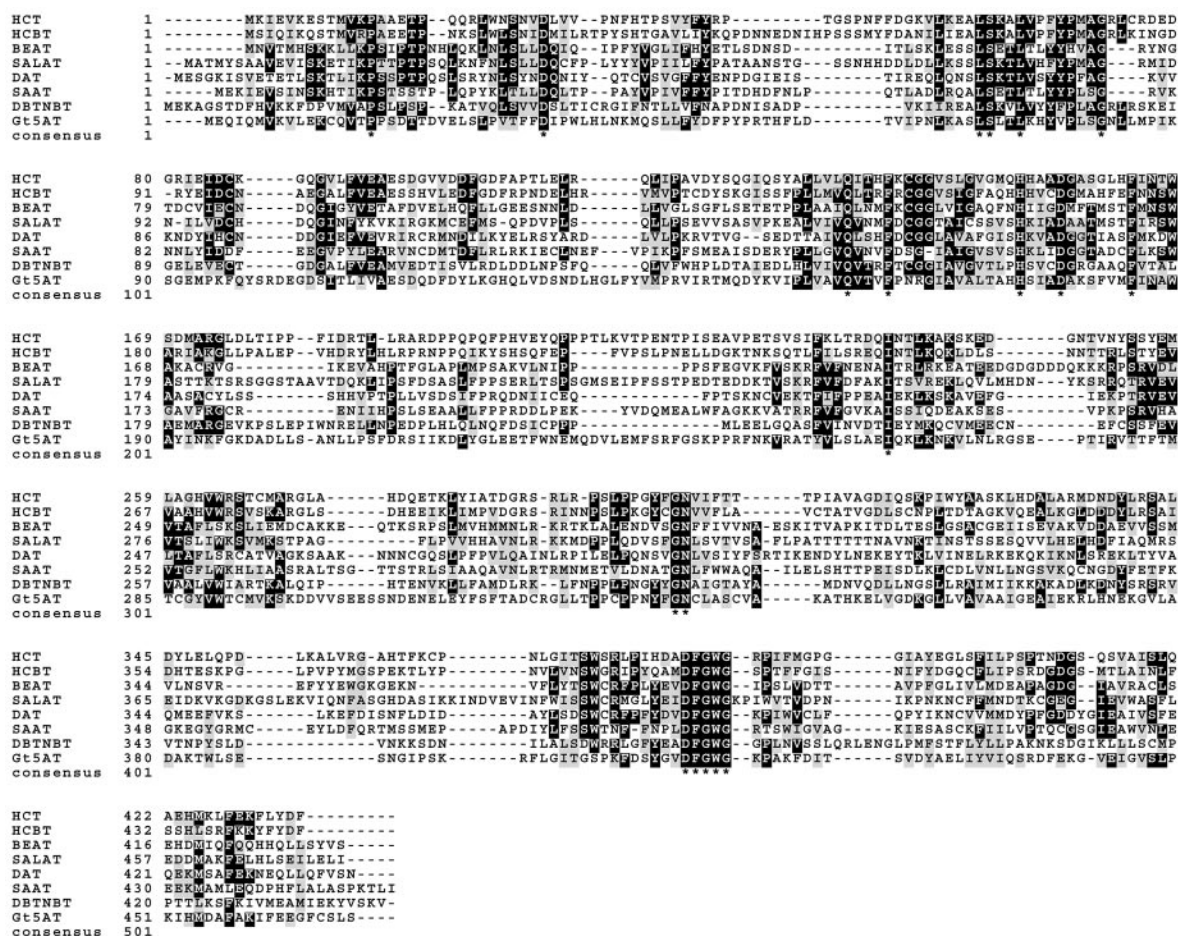


FIG. 6. Sequence alignment of representative members of the plant acyltransferase family. HCT was from *N. tabacum* (this work). HCBT, anthranilate *N*-hydroxycinnamoyl/benzoyltransferase from *Dianthus caryophyllus*; BEAT, benzyl-alcohol acetyltransferase from *Clarkia breweri*; SALAT, salutaridinol 7-*O*-acetyltransferase from *Papaver somniferum*; DAT, deacetylindoline 4-*O*-acetyltransferase from *Catharanthus roseus*; SAAT, strawberry alcohol acyltransferase from *Fragaria ananassa*; DBTNBT, 3'-*N*-debenzoyl-2'-deoxytaxol *N*-benzoyltransferase from *Taxus cuspidata*; Gt5AT, anthocyanin-5-*O*-glucoside 6'-*O*-acyltransferase from *Gentiana triflora*. Black and gray boxes indicate conserved and similar residues, respectively, in at least four of the eight sequences shown. In the consensus row, asterisks indicate residues conserved in the eight sequences. GenBankTM/EBI accession numbers are given in the legend to Fig. 9.

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 $V_{\max}/K_m$ 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

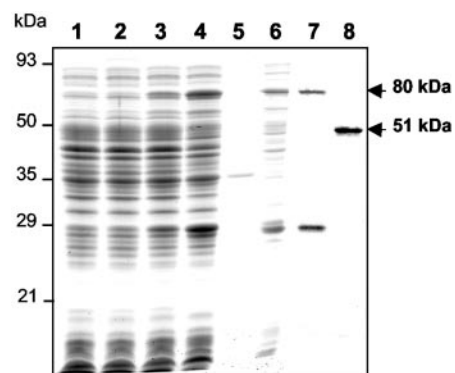


FIG. 7. Purification of the recombinant protein expressed in *E. coli*. Bacterial proteins were analyzed by SDS-PAGE and Coomassie Blue staining. Protein contents of uninduced (lane 1) and induced (lane 2) non-transformed cells were compared with those of uninduced (lane 3) and induced (lane 4) transformed cells. The GST-HCT fusion protein (80-kDa protein) strongly accumulated after induction of transformed bacteria (lane 4). The fusion protein was not detected in the insoluble fraction (lane 5), but was present in a large amount in the soluble fraction (lane 6). After fixation on glutathione-agarose beads, the fusion protein was either eluted with a glutathione solution (lane 7) or cleaved by thrombin to liberate the purified HCT protein (lane 8). The arrows indicate the positions of the GST-HCT fusion protein (80 kDa) and HCT (51 kDa). The positions of markers of known molecular masses are indicated on the right.

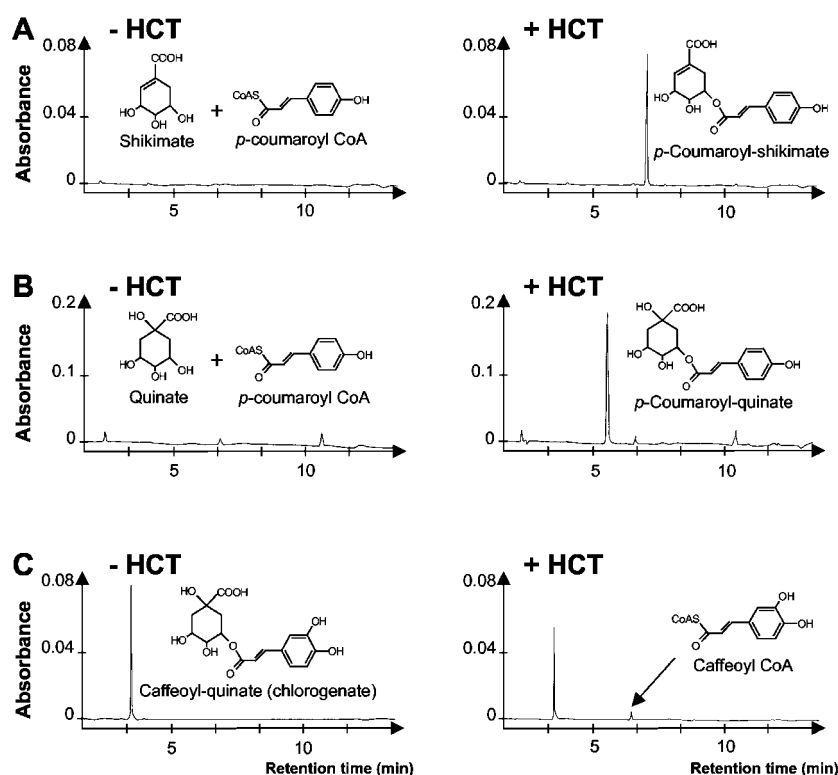


FIG. 8. HPLC analysis of HCT reaction products. An aliquot of the incubation medium without (left panels) or with (right panels) HCT was analyzed. The nature of the substrates tested is presented in the left panels, and that of the reaction products detected at 320 nm in the right panels. Products were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

TABLE II
Kinetic parameters of recombinant HCT

Concentrations of substrates and assay conditions are detailed under "Experimental Procedures." The K_m and V_{max} values were calculated from triplicates by the Lineweaver-Burk method.

Varying substrate	Saturating substrate (4 mM)	Kinetic parameters		
		K_m μM	V_{max} picokatal/mg	V_{max}/K_m picokatal/mg/ μM
<i>p</i> -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
<i>p</i> -Coumaroyl-CoA	Quinate	150 ± 50	4.6 ± 1.6	0.03
Caffeoyl-CoA	Quinate	730 ± 200	1.4 ± 0.35	0.002
Shikimate	<i>p</i> -Coumaroyl-CoA	750 ± 100	140 ± 20	0.19
Quinate	<i>p</i> -Coumaroyl-CoA	$70.10^3 \pm 40.10^3$	21 ± 10	0.0003

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.

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

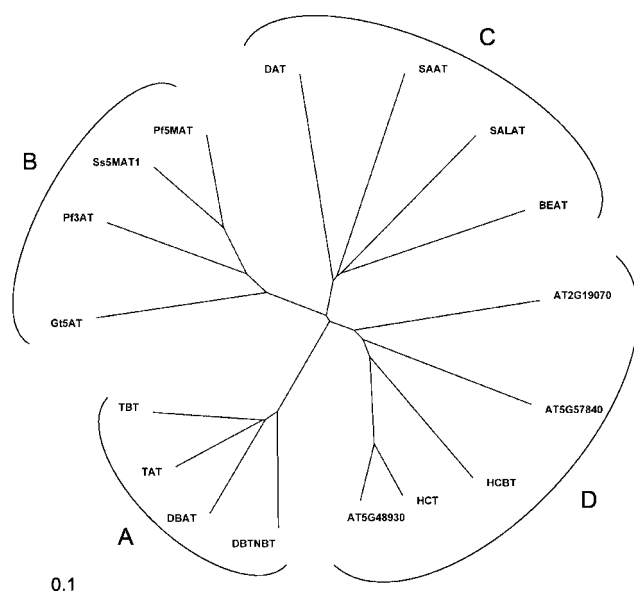


FIG. 9. Phylogenetic tree of acyltransferases revealing four evolutionary sequence clusters. The tree was constructed by neighbor-joining distance analysis. Line lengths indicate the relative distances between nodes. Sequences of biochemically characterized enzymes were used for alignment, *DBTNBT*, 3'-*N*-debenzoyl-2'-deoxytaxol *N*-benzoyltransferase from *T. cuspidata* (GenBank™/EBI accession number AF466397); *DBAT*, deacetylbaecatin III 10-*O*-acetyltransferase from *T. cuspidata* (accession number AF193765); *TAT*, taxadienol acetyltransferase from *T. cuspidata* (accession number AF190130); *TBT*, taxane benzoyltransferase from *T. cuspidata* (accession number AF297618); *Gt5AT*, anthocyanin-5-*O*-glucoside 6"-*O*-acyltransferase from *G. triflora* (accession number BAA74428); *Pf3AT*, anthocyanin-3-*O*-glucoside 6"-*O*-acyltransferase from *P. frutescens* (accession number BAA93475); *Ss5MAT1*, anthocyanin-5-*O*-glucoside 6"-*O*-malonyltransferase from *Salvia splendens* (accession number AF405707); *Pf5MAT*, anthocyanin 5-*O*-glucoside-6"-*O*-malonyltransferase from *Perilla frutescens* (accession number AF405204); *DAT*, deacetylindoline 4-*O*-acetyltransferase from *C. roseus* (accession number AF053307); *SAAT*, strawberry alcohol acyltransferase 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 AF043464); *AT2G19070* and *AT5G57840*, *A. thaliana* genes encoding putative acyltransferases; *HCBT*, anthranilate *N*-hydroxycinnamoyl/benzoyltransferase from *D. caryophyllus* (accession number Z84383); *AT5G48930*, *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).

shikimate pathway (*viz.* shikimate, quinate, and anthranilate). A survey of the *Arabidopsis* genome has uncovered ~60 genes putatively encoding acyltransferases of the same family.² Among them, three are located in group D, and the closest gene (*AT5G48930*) (Fig. 9) codes for an acyltransferase with biochemical activity similar to that of HCT.³ Thus, phylogenetic analysis of the acyltransferase family defines four evolutionary groups and should provide a useful framework for studying the functions of the numerous members of this plant gene family.

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

Phenylalanine

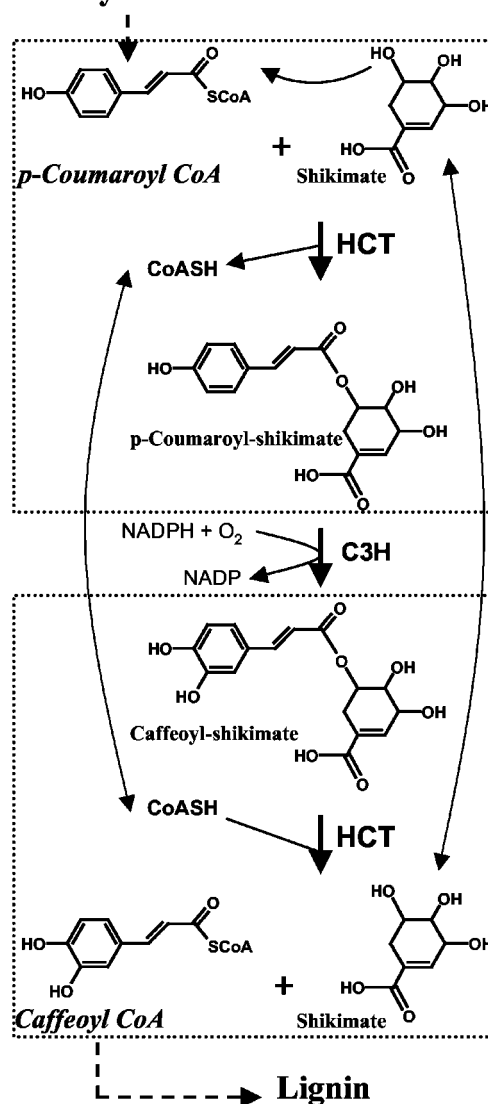


FIG. 10. Scheme of the function of HCT in the phenylpropanoid pathway. The steps catalyzed by HCT are boxed. The double-headed curved arrows illustrate CoASH and shikimate recycling. C3H, *p*-coumarate 3-hydroxylase.

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 feruloyl-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 Nicotianaceae, 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.

that 5-*O*-caffeoylquinic 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 caffeoylquinic 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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REFERENCES

1. Dixon, R. A., and Paiva, N. L. (1995) *Plant Cell* **7**, 1085–1097
2. Douglas, C. J. (1996) *Trends Plant Sci.* **1**, 171–178
3. Hahlbrock, K., and Scheel, D. (1989) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **40**, 347–369

4. Baucher, M., Monties, B., Van Montagu, M., and Boerjan, W. (1998) *Crit. Rev. Plant Sci.* **17**, 125–197
5. Boudet, A. M., Lapierre, C., and Grima-Pettenati, J. (1995) *New Phytol.* **129**, 203–236
6. Petersen, M., Strack, D., and Matern, U. (1999) *Annu. Plant Rev.* **2**, 151–221
7. Zenk, M. H. (1979) *Recent Adv. Phytochem.* **12**, 139–176
8. Allina, S. M., Pri-Hadash, A., Theilmann, D. A., Ellis, B. E., and Douglas, C. J. (1998) *Plant Physiol.* **116**, 743–754
9. Lee, D., and Douglas, C. J. (1996) *Plant Physiol.* **112**, 193–205
10. Maury, S., Geoffroy, P., and Legrand, M. (1999) *Plant Physiol.* **121**, 215–224
11. Ye, Z.-H., Kneusel, R. E., Matern, U., and Varner, J. E. (1994) *Plant Cell* **6**, 1427–1439
12. Humphreys, J. M., Hemm, M. R., and Chapple, C. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 10045–10050
13. Osakabe, K., Tsao, C. C., Li, L., Popko, J. L., Umezawa, T., Carraway, D. T., Smeltzer, R. H., Joshi, C. P., and Chiang, V. L. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 8955–8960
14. Chen, F., Yasuda, S., and Fukushima, F. (1999) *Planta* **207**, 597–603
15. Schoch, G., Goepfer, S., Morant, M., Hehn, A., Meyer, D., Ullmann, P., and Werck-Reichhart, (2001) *J. Biol. Chem.* **276**, 36566–36574
16. Franke, R., Hemm, M. R., Denault, J. W., Ruegger, M. O., Humphreys, J. M., and Chapple, C. (2002) *Plant J.* **30**, 47–59
17. Franke, R., Humphreys, J. M., Hemm, M. R., Denault, J. W., Ruegger, M. O., Cusumano, J. C., and Chapple, C. (2002) *Plant J.* **30**, 33–45
18. Yang, Q., Rheihard, K., Schlitz, E., and Matern, U. (1997) *Plant Mol. Biol.* **35**, 777–789
19. St. Pierre, B., Laflamme, P., Alarco, A., and De Luca, V. (1998) *Plant J.* **14**, 703–713
20. Martz, F., Maury, S., Pinçon, G., and Legrand, M. (1998) *Plant Mol. Biol.* **36**, 427–437
21. Legrand, M., Kauffmann, S., Geoffroy, P., and Fritig, B. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84**, 6750–6754
22. Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
23. Negrel, J., and Smith, T. A. (1984) *Phytochemistry* **23**, 31–34
24. Lüderitz, T., Shatz, G., and Grisebach, H. (1982) *Eur. J. Biochem.* **123**, 583–586
25. Strack, D., and Gross, W. (1990) *Plant Physiol.* **92**, 41–47
26. Legrand, M., Fritig, B., and Hirth, L. (1978) *Planta* **144**, 101–108
27. Stöckigt, J., and Zenk, M. H. (1975) *Z. Naturforsch. Sect. C Biosci.* **30**, 352–358
28. Rhodes, M., and Woollorton, L. (1976) *Phytochemistry* **15**, 947–951
29. Rhodes, M., Woollorton, L., and Lourenço, E. (1979) *Phytochemistry* **18**, 1125–1129
30. Ulbrich, B., and Zenk, M. (1979) *Phytochemistry* **18**, 929–933
31. Ulbrich, B., and Zenk, M. (1980) *Phytochemistry* **19**, 1625–1629
32. Walker, K., Long, R., and Croteau, R. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99**, 9166–9171
33. Humphreys, J. M., and Chapple, C. (2002) *Curr. Opin. Plant Biol.* **5**, 224–229
34. Herrmann, K. M. (1995) *Plant Cell* **7**, 907–919
35. Legrand, M., Fritig, B., and Hirth, L. (1971) *C. R. Acad. Sci. Paris Ser. D* **273**, 525–528
36. Stöckigt, J., and Zenk, H. (1974) *FEBS Lett.* **42**, 131–134
37. Fritig, B., Legrand, M., and Hirth, L. (1972) *Virology* **47**, 845–848
38. Pinçon, G., Maury, S., Hoffmann, L., Geoffroy, P., Lapierre, C., Pollet, B., and Legrand, M. (2001) *Phytochemistry* **57**, 1167–1176
39. Atanassova, R., Favet, N., Martz, F., Chabbert, B., Thollier, M. T., Monties, B., Fritig, B., and Legrand, M. (1995) *Plant J.* **8**, 465–477