Characterization of Hydroxycinnamoyl-coenzyme A Shikimate Hydroxycinnamoyltransferase from *Populus euramericana*

Bong-Gyu Kim, In-Ah Kim, and Joong-Hoon Ahn*

Department of Bioscience and Biotechnology, Bio/Molecular Informatics Center, Konkuk University, Seoul 143-701, Republic of Korea

Hydroxycinnamoyl-coenzyme A shikimate/quinate hydroxycinnamoyltransferase (HCT), *PeHCT* was cloned from *Populus euramericana*. The predicted amino acid sequence revealed that PeHCT contained a conserved HXXXDG motif that was found in acyltransferase. The *in vitro* substrate was determined using recombinant PeHCT. The recombinant PeHCT showed substrate-specificity towards shikimic acid. In addition, *PeHCT* displayed higher expression in the stem than in the leaf, and its expression increased during the growth stage of the leaf. Results indicate PeHCT could be involved in lignin biosynthesis by formation of *p*-coumaroyl shikimate.

Key words: hydoxylcinnamate, hydroxycinnamoyl-coenzyme A shikimate hydroxycinnamoyltransferase, *Populus euramericana*

Plants produce a variety of phenolic compounds through the phenylpropanoid pathway. The key enzyme located at the branch point between primary and secondary metabolisms is phenylalanine ammonia lyase (PAL). The first reaction product of PAL is cinnamic acid. Hydroxylation and *O*-methylation of cinnamic acid lead to the formation of hydroxylcinnamates including *p*-coumaric acid (PA), caffeic acid (CA), ferulic acid (FA), and sinapic acid (SA). Hydroxylcinnamates are conjugated with CoA by coumaroyl-CoA ligase (4CL). The CoA esters of hydroxycinnamates serve as substrates for the biosyntheses of flavonoids, isoflavonoids, anthocyanins, stilbenes, lignans, lignins, as well as other esters and amides [Dixon and Paiva, 1995]. In the process of lignin biosynthesis, cinnamoyl-CoA forms conjugates with quinic acid and/or shikimic acid [Clifford, 1999]. Cinnamoylquinnate or cinnamoylshikimate serves as a substrate for 3-hydroxylation by *p*-coumarate 3-hydroxylase (C3H), whereas cinnamic acid and cinnamoyl-CoA are not substrates for C3H [Schoch *et al*., 2001]. The mutation of C3H in *Arabidopsis thaliana* affected the biosynthesis of lignin [Franke *et al*., 2002a; 2002b]. Thus, the formation of cinnamoylquinnate or cinnamoylshikimate

is important for the lignin biosynthesis. The silencing of hydroxycinnamoyl-coenzyme A shikimate/quinate hydroxycinnamoyltransferase (HCT) in tobacco resulted in changes in the lignin composition [Hoffman *et al*., 2004]. In addition, the accumulation of hydroxylcinnamoylquinnate conjugates showed resistance to infection by a bacterial pathogen as well as improved antioxidant capacity [Niggeweg *et al*., 2004].

Formation of hydroxycinnamoylquinnate or hydroxycinnamoylshikimate is mediated by HCT. HCT is a member of the BAHD [BEAT (benzylalcohol *O*-acetyltransferase)] family acyltransferases. AHCT (anthocyanin *O*-hydroxycinnamoyltransferase), HCBT (anthranilate *N*-hydroxycinnamoyl/bezoyltransferase), DAT (deacetylvindoline 4-*O*-acetyltransferase) [St Pierre and De Luca, 2000]. The first gene for HCT was cloned from tobacco [Hoffman *et al*., 2003]. Thereafter, the *in vivo* function of HCT in *A. thaliana*, *Nicotiana tabacum*, and *Pinus radiate* was determined [Hoffman *et al*., 2004; Wagner *et al*., 2007]. However, only a few studies on the enzymatic characterization of HCT have been reported. In the present study, the cloning and characterization of HCT from *Populus euramericana* (*PeHCT*) were evaluated, and results showed that PeHCT encodes a hydroxycinnamoyl-coenzyme A shikimate hydroxycinnamoyltransferase and is likely to be involved in lignin biosynthesis.

Poplar gene index at the Dana-Farber Cancer Center (http://compbio.dfci.harvard.edu/cgi-bin/tgi/gimain.pl?gudb

*Corresponding author
Phone: +82-2-450-3764; Fax: +82-2-3437-6106
E-mail: jhahn@konkuk.ac.kr

Table 1. List of primers

Gene name	Poplar Gene index number	Forward primer	Reverse Primer
PeHCT-1	gi|224115831	ATGATAATCAACGTGAAGGAGTCAACC	ATGCGGCCGCTTAAATGTCATATATGAACT
PeHCT-2	gi|224072740	ATGCTACCTCTTCATAGAACTTGGGGT	ATGCGGCCGCTCATTCTTTAATGTCATATA
PeHCT-3	gi|224142374	ATGAAAGTTGATGTGAAACAGTCAACT	ATGCGGCCGCCTAGAAATCGTACAGGCA
PeHCT-4	gi|224142366	ATGCAGATTACCGTAAAGGAATCA	ATGCGGCCGCTTACAGGCAGCTCCGTTTAT
PeHCT-5	gi|224123767	ATGCAGATGGTAAGAGTTGAAATTCGAA	ATGCGGCCGCTTAGAAATCGTAAAAGGAC
PeHCT-6	gi|224126144	ATGAAAATTTACTTGTCACTTTGCTCT	ATGCGGCCGCTTATATATCTTCATAGAAGT
PeHCT-7	gi|224134055	ATGGCAGATGGTAGTAACGATGCTT	ATGCGGCCGCTCAAATCTGCATTAGTTCT

=poplar) was examined to find HCT homologues, and seven HCT homologues were found (Table 1). Primers for each HCT homologue were designed, and reverse-transcription polymerase chain reaction (RT-PCR) was conducted to clone each homologue. Total RNA was isolated from leave of poplar, which was grown at Konkuk University (Seoul, Korea), and RT was carried out using Omniscript reverse transcriptase (Qiagen, Hilden, Germany). PCR was performed using Hotstart Polymerase (Qiagen) at the following conditions: 40 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1.5 min. However, only one PCR product was obtained, which was then subcloned into pGEM-T (Promega, Madison, WI). The open reading frame (ORF) of *PeHCT* consisted of 1,296 bp encoding a 47.9-kDa protein. PeHCT also contained the conserved motif HXXXDG from amino acid 53 to 58, in which histidine residue served as a base for the reaction, and aspartic acid stabilized protonated histidine [D'Auria, 2006].

The ORF of *PeHCT* was subcloned into the *Eco*RI/*Not*I site of the *E. coli* expression vector pGEX 5X-1 (Amersham, Piscataway, NJ). The resulting construct was transformed into *E. coli* BL21 (DE3). The expressed recombinant PeHCT was purified using glutathione *S*-transferase (GST)-affinity chromatography. The molecular weight of the purified recombinant PeHCT was about 70 kDa, because the GST was connected to PeHCT (Fig. 1). In order to determine the substrate of PeHCT, 12.5 µg purified enzyme, 300 µM acetyl group acceptor (quinic acid or shikimic acid), and 60 µM *p*-coumaroyl-CoA were mixed in 200 µL of 100 mM Tris/HCl buffer (pH 8.0). *p*-Coumaroyl-CoA was synthesized using 4-coumaroyl-CoA ligase from rice (R4CL) [Lee *et al.*, 2007]. The reaction mixture was incubated for 1 h at 37°C. The reaction was then terminated by boiling the reaction mixture for 5 min, after which the proteins were precipitated by spinning the tube for 15 min at 13000 rpm, and the supernatant was recovered. The supernatant was analyzed via high performance liquid chromatography spell out (HPLC) (Varian, Santa Clara, CA) equipped
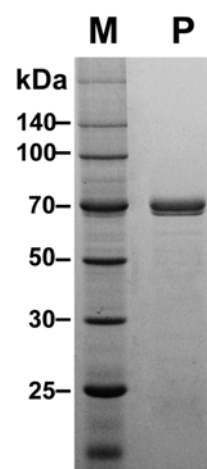

Fig. 1. Expression and purification of the recombinant PeHCT. M, Molecular weight marker; P, purified recombinant PeHCT

with a photodiode array detector using a Varian polar C18 reversed-phase column (Varian; 4.60×250 mm, 0.45 µm). For analytical scale, the mobile phase consisted of H_2O containing 0.1% formic acid (pH 3.0) programmed as follows: 15% acetonitrile at 0 min, 20% acetonitrile at 10 min, 40% acetonitrile at 15 min, 90% acetonitrile at 20 min, 90% acetonitrile at 25 min, 15% acetonitrile at 25.1 min, and 15% acetonitrile at 50 min. The flow rate was 1 mL/min and UV detection was dually performed at 290 and 320 nm. The HPLC analysis of the shikimic acid reaction product with PeHCT showed several peaks. These peaks were not observed in a negative control, which did not contain the recombinant PeHCT. Mass spectroscopy analysis of these reaction products revealed that these products were divided into two groups. The molecular mass of the first group (P1-P3 in Fig. 2B) was 320 Da (Fig. 2C), and the molecular mass of the second group (P4-P6 in Fig. 2B) was 466 Da. Based on the molecular mass and fragmentation pattern, reaction products in the first group found to have one coumaric acid attached to skimic acid. Shikimic acid had three hydroxyl groups, each of which reacted with *p*-coumaroyl-
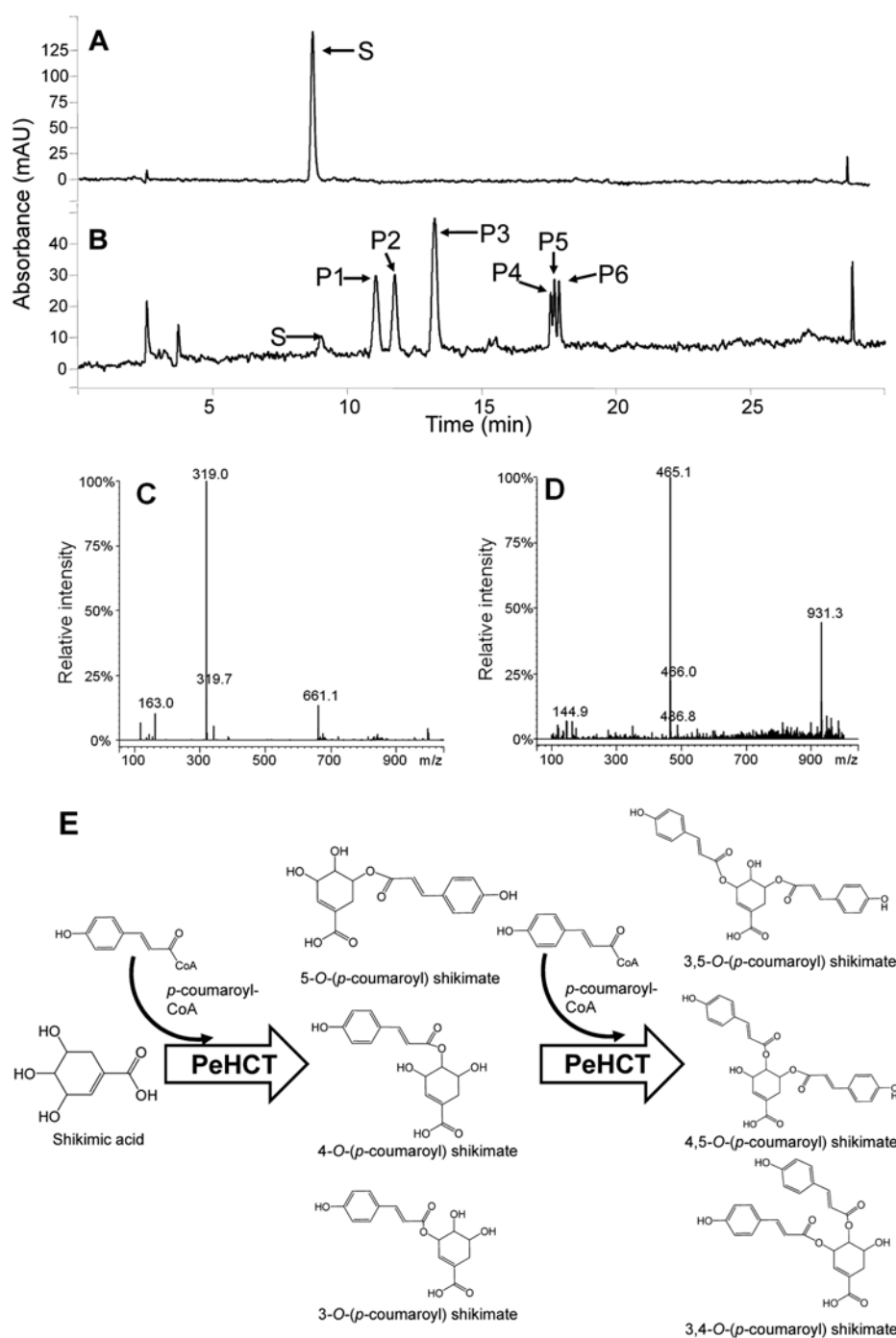
Fig. 2. HPLC analysis of reaction products produced by reaction with recombinant PeHCT, shikimic acid, and *p*-coumaroyl-CoA. S, *p*-coumaroyl-CoA; P1-P6, reaction products. A, authentic *p*-coumaroyl-CoA; B, HPLC profile of the reaction product; C, Ionization tandem mass spectrometry (ESI-MS/MS) analysis spectrum of product 1 (P1) in negative mode; D, ESI-MS/MS analysis spectrum of product 2 (P4) in negative mode. Products 1, 2, and 3 (P1-P3) showed the same molecular weight as revealed by MS/MS analysis. Products 4, 5, and 6 (P4-P6) also showed the same molecular weight by MS/MS analysis.; E, Reaction scheme of PeHCT with shikimic acid and *p*-coumaroyl-CoA

CoA, resulting in three different reaction products. Based on the results of Schoch *et al*. [2006], P1 was determined as 3-*O*-(*p*-coumaroyl) shikimic acid, P2 as 4-*O*-(*p*-coumaroyl) shikimic acid, and P3 as 3-*O*-(*p*-coumaroyl) shikimic acid. In the second group (P4-P6), two molecules of coumaric acid were attached to shikimic acid in different positions (Fig. 2E). Taken together, these results showed that the esterification reaction between *p*-coumaroyl-CoA and shikimic acid occurred. In addition, quinic acid also served as a substrate for PeHCT.

Table 2. Kinetic parameters of recombinant PeHCT

Varying substrate	Saturating substrate	K_m ($\times 10^3$ nM)	V_{max} (nM/min)	V_{max}/K_m (min^{-1})
p-coumaroyl-CoA	Shikimic acid	8125	1251338	1.54×10^{-2}
p-coumaroyl-CoA	Quinic acid	9635	740197	0.77×10^{-2}
Shikimic acid	*p*-coumaric acid	895	614106	0.68×10^{-2}
Quinic acid	*p*-coumaric acid	5819	2507	0.43×10^{-2}

The values were calculated from triplicate tests using the Lineweaver-Burk method.
Five micrograms of the purified recombinant PeHCT and 500 µM of saturating substrate were used. The concentration of varying substrate was from 10 to 250 µM. Reaction was carried out at 37°C for 20 min.
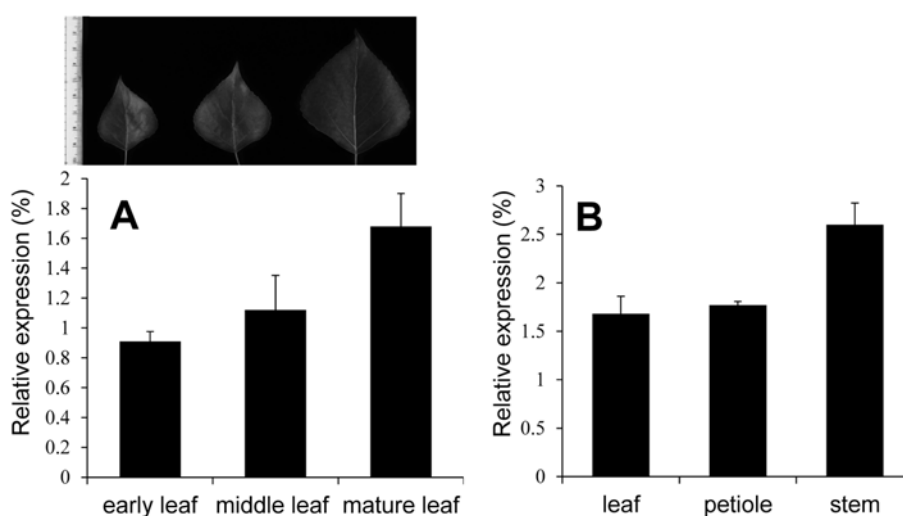

Fig. 3. Relative expression of *PeHCT* during different growth stages (A) and different tissues (B). Total RNA was extracted from leaf tissue at three different growth stages, petiole and stem. Three different stages of leaf are shown in A. Relative transcript levels were standardized to the constitutively expressed actin gene. Each data point is the average of three replicate PCR reactions with the standard error of the mean normalized expression level indicated by error bars.

In order to determine the substrate preference of PeHCT, the kinetic parameters, K_m and V_{max} were determined using the Lineweaver-Burk method towards the acyl group donor (*p*-coumaroyl-CoA) and the acyl group acceptor (shikimic acid and quinic acid). Shikimic acid was a superior substrate compared to quinic acid (Table 1). Poplar actively synthesizes lignin like other tress. The PeHCT reaction product, *p*-coumaroyl shikimate ester was transformed caffeoyl shikimate by the action of C3H, which led to the synthesis of gualacyl lignin (G-lignin) and syringyl lignin (S-lignin) [Schoch *et al.*, 2001]. To predict the *in vivo* function of PeHCT, the expression of *PeHCT* was examined. The expression of *PeHCT* during different leaf growth stages and in different tissues (leaf, stem, and petiole) was analyzed by real time quantitative PCR (qPCR). Leaves were collected at three different growth stages (Fig. 3A). Petioles and stems, which were connected to the mature leave, were also collected. Total RNA from each sample was isolated using Plant total RNA isolation kit (Qiagen). qPCR was carried out as described by Kim *et al.* [2007].

Overall, the expression of *PeHCT* was comparable to that of actin, which was used for normalization. Expression of *PeHCT* increased as the leaf size increased (Fig. 3A). Mature leaves contained more lignin than younger ones. Thus, this result indicates that the expression of *PeHCT* is correlated with the lignin content. Stem contains more lignin than leaf. Thus, expression of *PeHCT* in the stem would be higher than that of leaf when *PeHCT* is involved in lignin biosynthesis. The stem exhibited approximately 1.5-fold more expression of *PeHCT* than the leaf (Fig. 3B). Taken together, these results, along with the substrate preference of PeHCT, suggest that *PeHCT* is likely to be involved in the lignin biosynthesis pathway through the formation of *p*-coumaroyl shikimate.

Four HCTs were biochemically characterized [Hoffmann *et al.*, 2003; Niggeweg *et al.*, 2004; Sonnante *et al.*, 2010]. Two hydroxycinnamoyl-CoA shikimate/quinate transferases (HS/QHTs) from tobacco showed distinct acyl-acceptor specificity, and the first one showed a greater preference towards shikimic acid, indicating that it may be involved in lignin biosynthesis. In addition, the

other preferred quinic acid, indicating it may mediate the chlorogenic acid biosynthesis. On the other hand, hydroxycinnamoyl-CoA quinate transferase (HQT1) and HQT2 from artichoke showed greater preference towards quinic acid than to shikimic acid. PeHCT was the first HCT from poplar to be biochemically characterized.

References

Clifford MN (1999) Chlorogenic acids and other cinnamtes-nature, occurrence and dietary burden. *J Sci Food Agric* **79**, 362-372.

D'Auria JC (2006) Acyltransferases in plants: a good time to be BAHD. *Cur Opin Plant Biol* **9**, 331-340.

Dixon RA and Paiva NL (1995) Stress-induced phenylpropanoid metabolism. *Plant Cell* **7**, 1085-1097.

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

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

Hoffmann L, Besseau S, Geoffroy P, Ritzenthaler C, Meyer D, Lapierre C, Pollet B, and Legrand M (2004) Silencing of hydroxycinnamoyl-coenzyme A shikimate/quinate hydroxycinnamoyltransferase affects phenylpropanoid biosynthesis. *Plant Cell* **16**, 1446-1465.

Hoffmann L, Maury S, Martz F, Geoffroy P, and Legrand M (2003) Purification, cloning, and properties of an acyltransferase controlling shikimate and quinate ester intermediates in phenylpropanoid metabolism. *J Biol Chem* **278**, 95-103.

Kim BG, Kim JH, Min SY, Shin KH, Kim JH, Kim HI, Ryu SN, and Ahn JH (2007) Anthocyanin content in rice is related to expression level of anthocyanin biosynthetic genes. *J Plant Biol* **50**, 156-160.

Lee YJ, Jeon Y, Lee JS, Kim BG, Lee CH, and Ahn J-H (2007) Enzymatic synthesis of phenolic CoAs using 4-coumarate:coenzyme A ligase (4CL) from rice. *Bull Korean Chem Soc* **28**, 365-366.

Niggeweg R, Michasel AJ, and Martin C (2004) Engineering plants with increased levels of the antioxidant chlorogenic acid. *Nat Biotechnol* **22**, 746-754.

Schoch G, Goepfert S, Morant M, Hehn A, Meyer D, Ullmann P, and Werck-Reichhart D (2001) CYP98A3 from *Arabidopsis thaliana* is a 3'-hydroxylase of phenolic esters, a missing link in the phenylpropanoid pathway. *J Biol Chem* **276**, 36566-36574.

Schoch GA, Morant M, Abdulrazzak N, Asnaghi C, Goepfert S, Peterson M, Ullmann P, and Werck-Reichhart D (2006) The *meta*-hydroxylation step in the phenylpropanoid pathway: A new level of complexity in the pathway and its regulation. *Environ Chem Lett* **4**, 127-136.

Sonnante G, D'Amore R, Blanco E, Pierri CL, De Palma M, Luo J, and Tucci Mand Martin C (2010) Novel hydroxycinnamoyl-coenzyme A quinate transferase genes from artichoke are involved in the synthesis of chlorogenic acid. *Plant Physiol* **153**, 1224-1238.

St Pierre B and De Luca V (2000) Evolution of acyltransferase genes: Origin and diversification of the BAHD superfamily of acyltransferases involved in secondary metabolism. In *Recent Advances in Phytochemistry* vol. 34. Romeo JT, Ibrahim R, Varin L, and De Luca V (eds), pp. 285-315, Elsevier Science, Amsterdam, Netherlands.

Wagner A, Ralph J, Akiyama T, Flint H, Phillips L, Torr K, Nanayakkara B, and Kiri LT (2007) Exploring lignification in conifers by silencing hydroxycinnamoyl-CoA:shikimate hydroxycinnamoyltransferase in *Pinuc radiata*. *Proc Natl Aca Sci USA* **104**, 11856-11861.