

# Molecular Cloning and Characterization of Phenylalanine Ammonia-lyase and Cinnamate 4-Hydroxylase in the Phenylpropanoid Biosynthesis Pathway in Garlic (*Allium sativum*)

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The cDNAs encoding phenylalanine ammonia-lyase (PAL) and cinnamate 4-hydroxylase (C4H) were cloned from garlic (*Allium sativum*) using reverse transcription-polymerase chain reaction (RT-PCR) with degenerate primers and 5' and 3' rapid amplification of cDNA ends (RACE) PCR. Amino acid sequence alignments showed that AsPAL and AsC4H have more than 70% amino acid identity with their homologues in other plants. The expression of AsPAL and AsC4H transcripts was highest in the roots but surprisingly low in the bulbils, where phenylpropanoid compounds are most concentrated. These results suggest that some phenylpropanoids are synthesized in the roots and subsequently transported to the bulbils of *A. sativum*.

KEYWORDS: Phenylalanine ammonia-lyase (PAL); cinnamate 4-hydroxylase (C4H); phenylpropanoid biosynthesis; garlic (*Allium sativum*)

## INTRODUCTION

Garlic (*Allium sativum*), a member of the onion family (Alliaceae), has been used for more than 4000 years for both culinary and medicinal purposes (1). In fact, it is one of the earliest documented examples of plants used for therapeutic purposes (2,3). Garlic has many medicinal properties, including antioxidant, anticancer, hepatoprotective, immunomodulatory, and cardioprotective effects (4–6), because of the presence of over 2000 biologically active compounds (7). In particular, phenolic compounds, such as phenylpropanoids, have strong antioxidant and antimicrobial effects (8).

In higher plants, the phenylpropanoid biosynthesis pathway produces many physiologically important metabolites, such as flavonoids, lignins, coumarins, phytoalexins, and stilbenes (9) (**Figure 1**), which are involved in plant development, mechanical support, and disease resistance (10, 11). Phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) is the first enzyme in the phenylpropanoid pathway and catalyzes the conversion of L-phenylalanine to *trans*-cinnamic acid (12). Next, *trans*-cinnamic acid is hydroxylated into *p*-coumaric acid by cinnamate 4-hydroxylase (C4H, CYP73, EC 1.14.13.11), a member of the cytochrome P450 superfamily (13). Subsequently, *p*-coumaric acid is transformed into phenylpropanoid compounds that are involved in diverse functions, such as

mechanical support, synthesis of anthocyanins, signaling via flavonoid nodulation factors, and protection against biotic and abiotic stresses (14-16). Research studies on PAL and C4H have important applications in biotechnology and elucidating the regulation of the phenylpropanoid biosynthesis pathway because they are involved in controlling its flux (17). For example, C4H controls the carbon flux for phytoalexins that are synthesized when plants are challenged by pathogens (18).

Although PAL has been cloned and sequenced from several species of plants, including *Salvia miltiorrhiza* (19), *Astragalus membranaceus* (12), *Ephedra sinica* (20), banana (21), and barley root (22), few studies have investigated PAL and C4H gene expression and phenylpropanoid biosynthesis in garlic (23). As a result, we cloned full-length cDNAs encoding PAL and C4H (GenBank accession numbers GU456381 and GU456382, respectively) from A. sativum and analyzed their expression levels by quantitative real-time polymerase chain reaction (PCR). We also determined the concentrations of various phenylpropanoid compounds in different organs of A. sativum by high-performance liquid chromatography (HPLC).

## MATERIALS AND METHODS

**Plant Material.** Garlic (*A. sativum*) was grown from bulbs in a greenhouse at the experimental farm of Chungnam National University (Daejeon, Korea). Plants were collected, then freeze-dried, and stored at -80 °C. Prior to experiments, each organ of *A. sativum* (e.g., bulbils, scapes, leaves, bulbs, and roots) was ground with a mortar and pestle under liquid nitrogen.

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Figure 1. Phenylpropanoid biosynthesis pathway in A. sativum. PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumaric acid coenzyme A ligase.

Total RNA Isolation and cDNA Library Synthesis. Total RNA was extracted from 100 mg of each powdered organ using TRIzol (Invitrogen, Carlsbad, CA). The RNA pellet was dissolved in diethylpyrocarbonate (DEPC)-treated water, and the quality and concentration of RNA were then determined by agarose gel electrophoresis and spectrophotometry, respectively. Subsequently, 1  $\mu$ g of total RNA was reverse-transcribed using the ReverTra Ace- $\alpha$  kit (Toyobo, Japan). The resulting cDNA was used as the template for quantitative real-time PCR and rapid amplification of cDNA ends (RACE) PCR.

**Isolation of cDNA Encoding AsPAL and AsC4H.** The GeneRacer kit (Invitrogen, Carlsbad, CA) was used to isolate the cDNA encoding AsPAL and AsC4H. All RACE primers were designed from the core sequences (**Table 1**). 5'-RACE PCR was performed using the GeneRacer 5' primer and the reverse primer of each gene. Likewise, 3'-RACE PCR was performed with the GeneRacer 3' primer and the forward primer of each gene. The TM Calculator program (http://www.genotech.co.kr) was used to compute the PCR annealing temperatures. The PCR products were cloned into the T-blunt vector (SolGent, Daejeon, Korea) and then sequenced by the National Instrumentation Center for Environmental Management (NICEM, Seoul National University, Seoul, Korea).

**Quantitative Real-Time PCR.** The gene expression levels of *AsPAL* and *AsC4H* were analyzed using a MiniOpticon real-time PCR detection system (BioRad, Hercules, CA). Primers were designed from the full-length cDNA sequences using the Primer3 program (http://frodo.wi.mit. edu/primer3) (**Table 1**). Real-time PCR was performed in a 20  $\mu$ L reaction volume with 0.4  $\mu$ M of each primer and 1× SYBR Green real-time PCR master mix (Toyobo). PCR protocols were as follows: 1 cycle of 5 min at 95 °C; 40 cycles with a denaturing time of 15 s at 95 °C, an annealing time of 15 s at 56 °C, and an elongation time of 20 s at 72 °C. Quantitative real-time PCR experiments were performed in triplicate. The actin gene from *A. sativum* (GenBank accession number AY821677) was used as an internal reference.

Sequence Analysis. Homologous sequences of AsPAL and AsC4H were identified using BLAST (http://www.ncbi.nlm.nih.gov/BLAST). Sequence alignments were constructed with the BioEdit Sequence

Alignment Editor, version 5.0.9 (Department of Microbiology, North Carolina State University, Raleigh, NC). Phylogenetic trees were constructed with TreeTop, Phylogenetic Tree Prediction (http://www.genebee. msu.su/services/phtree reduced.html).

HPLC Analysis of Methanolic Extracts of Garlic. Powdered garlic samples (0.2 g) were extracted with 3 mL of pure methanol at 60 °C for 1 h at room temperature. The extract was filtered through a 0.45  $\mu$ m poly filter (Acrodisc Syringe Filters, Pall, Port Washington, NY) and then diluted 2-fold with methanol. HPLC analysis was performed on a Futecs NS-4000 HPLC system (Daejeon, Korea) with a RS Tech C18 column (250 mm × 4.6 mm, 5  $\mu$ m, Daejeon, Korea). The column was maintained at 30 °C, and the mobile phase consisted of acetonitrile and 0.15% acetic acid. The flow rate was 1.0 mL/min, and the injection volume was 20  $\mu$ L. The concentrations of phenylpropanoid compounds were quantified using a standard curve. All samples were analyzed in triplicate.

### **RESULTS AND DISCUSSION**

**Cloning of PAL and C4H from** *A. sativum.* Reverse transcription (RT)-PCR generated a 1000 base pair (bp) fragment of *AsPAL* that was homologous with other plant *PAL* genes. Subsequently, 5'- and 3'-RACE PCR and end-to-end PCR yielded a 2124 bp open reading frame (ORF) encoding AsPAL. It has 708 amino acids, and its calculated molecular weight is 77 kDa.

Similarly, RT-PCR generated a 1080 bp fragment that was homologous with other plant *C4H* genes. Subsequently, 5'- and 3'-RACE PCR and end-to-end PCR produced a 1515 bp ORF encoding AsC4H. It has 507 amino acids, and its calculated molecular weight is 58.6 kDa.

Sequence Analyses of AsPAL and AsC4H. The multiple sequence alignment of PAL shows that AsPAL has 94% identity and 97% similarity with *Allium cepa* PAL, 78% identity and 87% similarity with *Arabidopsis thaliana* PAL, 79% identity and 87%

 Table 1. Primers Used for RACE PCR and Real-Time PCR

use		primer name	sequence (5	′ <b>-</b> 3′)	P	CR product	vector for cloning
		conserved PAL F	TMCARGGMTACTCHGO	CATMMG			
		conserved PAL_R	GCGCTYTNSACBTGGT	TNGTVA			
		conserved C4H_F	AACTGGCTBCABGTYG	GVGAYGAY			
		conserved C4H_R	ACCANGCTBTVACMAG	GATYTTG			
		GeneBacer F	CGACTGGAGCACGAG	CGACTGGAGCACCACGACACTGA			
RACE PCR		GeneRacer R	GCTGTCAACGATACGC	TACGTAACG	partial sequences of AsPAL or AsC4H		T-blunt
			GAAGTCGTTCACCAGCTCGGAGAAC				
			GACGTGTCAAGGAAAA	ACCCGTG			
			COTAGTGTCGTCTCG	ATCOCTOC			
				CCCCACACTT			
			GCCTCACCCTTCGAGG	TTOT			
				CTCC			
real-time PCR							
		C4H_R					
		Actin_F		GGTAGA			
		Actin_R	AGCICGIIGIAGAAAG	IGIGAI			
	AcDAL	NG	MAN TO	Panoceur DEUZDUUZZA			04
	ACPAL	MGA	VNGDFSVNNEIIRIEDPLNWGAAF	KAMSGSHLDEVKRMVNEN	REKSVALEGATLAVA	CVAAVAAG-EIKDVVLDECARDG	VK 84
	AtPAL1	MEINGAHKSNGGGVDAMI	.CG <mark>GDIKTKNMVINAE</mark> DPLNWGAA#	NE <mark>OMNGSHLDEVKRMVA</mark> EF	RKPVVNLGGDTLTIG	QVAAISTIGNSVKVƏLSETARAG	100 N 100
	BrPAL1 PtPAL2	MEVNGLSHGGEVDAMI MEFCODSCI	CGGEIK-KNATVVGADPLNWGAAF GNGSLGFNPNDPLNWGVM	ECMEGSHLDEVKRMVEE SINGSHLDEVKRMVEE	RRPVVNLEGETLITIG	QVAAISTLGNGVKVƏLSENARƏG QVNAIASRDVGVKVƏLSEƏARMG	VK 97
	AsPAL	ASSEWVMDSMOKGTESYG	WITGFGATSHRRIKNG ALONELI	RELNAGIEGS-PNSGNSI	PSTTSRAAMLVRMNT	MOGNEGHERRIMAN	183
	AcPAL	ASSEWVM <mark>I</mark> SM <mark>C</mark> KGTESYG	VTTGFGATSHRRTKNG <mark>V</mark> ALQ <mark>N</mark> ELI	IRFLNAGIFGS <mark>-PNSGNS</mark> I	lp <mark>sttt</mark> raamlvr <mark>v</mark> nt	LLQGYSGIRFEILESIT <mark>RL</mark> LN <sup>A</sup> N	IT 183
	AtPAL1	ASSEWVNOSMNKGTESYG	VTIGFGATSHRRTKNG <mark>V</mark> ALQ <sup>3</sup> ELI	IRFLNAGIFGSTKETSHT	LPHSATRAAMLVRINT	LLQGESGIRFEILENITSELNNN	IT 200
	PtPAL1 PtPAL2	ASSEWVMOSMCKGIESIG	WTTGFGATSHRRTK <mark>OG</mark> G <mark>D</mark> LQKELI	IRFLNAGIFG <mark>NGTESTHT</mark> I	LPHSATRAAMLVRINI LPHSASRAAMLVRINI	LLQGYSGIRFEILEAITSELNUN LLQGYSGIRFEILE <mark>AITKL</mark> LNIN	IT 186
	AsPAL	PCLFLRGTITASGDLVFI	SYIAALLIGRENSKLVISDNILL	ASEAFCLAGITSGFFRLC	PKEGLALVNGTAVGS	GIASIVLYEINVLAVLAEV SAL	C 283
	AcPAL	PCLFLRGTITASGDLVFI	SYIA <mark>A</mark> LLTGRFNSK <mark>SVTSDNTL</mark> LI	PSEAFCLAGISGFFCLC	PREGLALVNGTAVGS	GHASHVLYENNVLYVLAEVYSAH	FC 283
	AtPAL1	PSLPLRGTITASGDLVPI DSLPLRGTITASGDLVPI	SYIA CLITGRENSKATGPNGDAL	ADEAF&LAGISSGFFDLC	CEREGLALVNGTAVGS	GUASUVLEEINVLEVLAEIDSAV GUASUUT OFANUT SUTAFUT SAT	3A 300
	PtPAL2	POLFLEGTITASGDLVFI	SYIA GLLTGRENSKAVGENGET	AVEAFTLAGINGGFFPLC	PKEGLALVNGTAVGS	GTASMVLFEANVLAILSEVUSAI	EA 286
	AsPAL	EVMOGRPEFTCHLTHKLK	HHPGQIE <mark>W</mark> AAIMEHIL <mark>E</mark> GSSYMK	AKNUHDMDPLQKEKQDRY	ALRTSPOWLGPCIEV	IRATKSIEREINSVNCNPLICV	SR 383
	AcPAL	EVMOGRPEFTDHLTH	HHPGQIEAAAIMEHIL <sup>9</sup> GSSYMK	A & KLH <mark>DT</mark> DFLQKFKQDRY	ALRTSPOWLGPQIEV	IRNATKSIEREINSVNCNPLICV	SR 383
	ATPAL1 BrPAL1	EVMSGRPEFICHLINGLE	HHPGCIEAAAIMEHIL GSSYMK	ACKLHENDELCKEKCORY	ALRISEQWLGEQIEV	IRMATKSIEREINSVNUNPLIUV	SR 400
	PtPAL2	EVM <mark>O</mark> GKPEFTDHLTH <mark>K</mark> LK	HHPGCIEAAAIMEHIL <mark>D</mark> GSSY <mark>W</mark> K	ACKLHEIDPLCKFKCDRY	ALRTSPOWLGPLIEV	IR <mark>TS</mark> TKMIEREINSVNCNPLICV	SR 386
	AnDAL	NES TUCONECCEDITOURS	INTET & TARTOUTHEROEOGTUN	Symper news Techning	TUCEPOSETSUS OUC	OPT OPT ANDUT JULIORA FOUNOF	492
	ACPAL	NKANHGGNFCGTPIGVSP	INTRIAMAAIGKIMFAQFSELVNI	DYNNGLPSNLTGCRNPSI	LEYGFKGAEIAMASYC	SELOGLANPVINHVCSAECHNCD	VN 483
	AtPAL1	NKATHGGNFQGTPIGVSM	ENTRLATAAIGKLMFAQFSELVNI	FYNNGLFSNLT <mark>AS</mark> RNFSI	LDYGFKGAEIAMASYC	SELQYLANEVTSHVQSAEQHNQD	VN 500
	BrPAL1	NKATHGGNFQGTPIGVSM	ENTRLATAAIGKLMFAQFSELVNI	YYNNGLPSNLT <mark>AS</mark> RNPSI	LDYGFKGAEIAMASYC	SELQULANEVTSHVQSAEQHNQD	VN 497
	PEPALZ	NKAHGGNEGGIEIGVSN	INTRUATASIGKLMEAGESELVNI	MINNGLPSNLIGGRNP51	LEIGERGAEIAMASIC	SELQULANEVIZHVQSAEQHNQL	486
	AsPAL	SLGLIS <sup>M</sup> RKTDEAV <mark>M</mark> ILK	LMSTTFLVALCQAVDLRHLEENL	QWVKGVVSQAVKSVLTVG	SANGERHPSRFCEKCL	IKMIDRENVETYADDACSACYPL	MQ 583
	ACPAL	SLGLISARKTERAVIIL	IMSTIFLVALCQAIDLRHLEEN	QAVKNAVSQAAKSVLTM	SANGE HPSRFCEKDL	IKMIDREYVETYADDACSANYPL	M0 583
	BrPAL1	SLGLISSRKISLAVDIL	IMSTIFLVANCQAVELRHLEENL	ONVKNOVSOMAKAVLING	SWNGELHPSRFCERDL	KVWERE VNI IADDRCSARIPL	10 597
	PtPAL2	SIGLIS#RKT#EAV2ILF	IMSTTULVA CQAUDIRHUEENI	NTVKNUVSQVAKSVLTVO	JENGE <mark>l</mark> hpsrfcekdl	UKVVDREHAFTYUDDECSAUYPL	MC 586
	AsPAL	KARQVIVEHALCOMEREN	DAETSIF <mark>C</mark> KIGAFEEELK <mark>RT</mark> LPKE	VEAVEMAFDNGKOAMENE	RIRECRSYPLYR	ELG <mark>AGY</mark> LTGE <mark>EC</mark> TSPGE <mark>V</mark> FEKVF	DA 683
	AcPAL	KARQVIVEHALCNGESER	DSDISIF-KIGAFEEELK <mark>RI</mark> LPKE	VEVVRAAFDNGKCVLPNF	RISECRSYPLYR	ELG <mark>AGYL</mark> AGE <mark>EC</mark> TSFGEMF <mark>P</mark> KVF	DA 683
	AtPAL1	KURQVIVEHALINGESER	MANTSIFERIGAFEEELKANLPRE	VEAARAAYDNGIISAHENE	RIGECRSYPLYREVRE	ELGHEDLIGE KVISPGE »FOKVF FIGUELITGE KVISPGE »FOKVF	00 700 00 697
	PtPAL2	KURÇVUVEHALMNGEÇE	NSSTSIFCKIGAFEEELKTLLPKE	VENARLELPNGNEAIPNE	RITECRSYPLYKEVRE	ELGTILITGEKVGSPGEDFIKVF	TA 686
	AsPAL	VEREEVVERLIECEOR	GARMEIC 708				
	AcPAL	VCNGKVVDPLLECLCEWI	GAPIFIC 708				
	AtPAL1	ICEGKI IEPMMECLNEWN	GAPIFIC 725				
	PtPAL2	ICAGELIDPHLECLKEW	GAPT PLC 711				

Figure 2. Multiple sequence alignment of *A. sativum* PAL (AsPAL) and four homologues in other plants: *A. cepa* AcPAL (AY541031), *A. thaliana* AtPAL1 (AY079363), *B. rapa* BrPAL1 (EU402423), and *P. trichocarpa* PtPAL2 (XM\_002311977). GenBank accession numbers are indicated in parentheses. Shaded areas indicate positions in the alignment with identical amino acids. The open black box highlights the active site of PAL.

similarity with *Brassica rapa* PAL, and 78% identity and 87% similarity with *Populus trichocarpa* PAL (Figure 2). PAL sequences diverge in the N terminus (24) but share a highly conserved motif (GTITASGDLVPLSYIA) corresponding to the active site of PAL (25). Phylogenetic analysis of these sequences further clarified their evolutionary relationships. As shown in Figure 3, the phylogenic tree divides PAL sequences into dicot and monocot groups. Specifically, PAL sequences in the dicot group are more closely related to each other than

those in the monocot group, except for two plants in the same genus (*A. sativum* and *A. cepa*).

Likewise, the multiple sequence alignment of C4H shows that AsC4H has 92% identity and 94% similarity with *A. cepa* C4H and 80–83% identity and 89–92% similarity with other homologues (**Figure 4**). Furthermore, the phylogenic tree confirms that the AsC4H sequence is homologous to other C4H sequences (**Figure 5**). It also shows that C4H sequences in monocots and dicots are closely related, except for *O. sativa* C4H.



Figure 3. Phylogenic tree of AsPAL and some of its homologues: *A. thaliana* AtPAL1 (AY079363), *B. rapa* BrPAL1 (EU402423), *P. trichocarpa* PtPAL2 (XM\_002311977), *Lactuca sativa* LsPAL (AF299330), *Rudbeckia hirta* RhPAL (EF070337), *Catharanthus roseus* CrPAL (AB042520), *A. cepa* AcPAL (AY541031), *Zea mays* ZmPAL (NM\_001153961), and *Oryza sativa* OsPAL (NM\_001059859). GenBank accession numbers are indicated in parentheses.

AsC4H AcC4H PtC4H1 CaC4H GaC4H AtC4H	MƏLLILEK <mark>ƏLISI FƏ</mark> AITIAIVISKIRG <mark>R</mark> AFKIP GƏD ƏMPIFGNWLQVGDDINHRNITNIAKKFGD <mark>Ə</mark> FLIRMGQRNLVVVSSPDLARDV MƏLLILEKƏLISI FƏAITIAIVISKIRGKAFKIP GƏD ƏMPIFGNWLQVGDDINHRNIANFAKKFGD FLIRMGQRNLVVVSSPDLARDV MƏLLILEKƏLIGS FƏAITVAILVSKIRGKƏFKIP GƏD ƏMPVFGNWLQVGDDINHRNITDIAKKFGD MƏLLILEKƏLIAIFVAILVAILVSKIRGKƏFKIP GƏD ƏMPVFGNWLQVGDDINHRNITTIAKKFGD MƏLLILEKƏLIAIFVAIVIAIAVSKIRGKƏFKIP GƏD ƏMPVFGNWLQVGDDINHRNITTIAKKFGD MƏLLILEKƏLIAIFVAIVIAIAVSKIRGKƏFKIP GƏD ƏMPVFGNWLQVGDDINHRNITTIAKFGD MƏLLILEKƏLIAIFVAIVIAIAVSKIRGKƏFKIP GƏD ƏMPVFGNWLQVGDDINHRNITTIAKFGD MƏLLILEKƏLIAVFVAVINAIVISKIRGKƏFKIP GƏD ƏMPVFGNWLQVGDDINHRNITDIAKKFGD MƏLLILEKƏLIAVFVAVINAIVISKIRGKƏFKIP GƏD ƏMPVFGNWLQVGDDINHRNITDIAKKFGD MƏLLILEKƏLIAVFVAVINAIVISKIRGKƏFKIP GƏD ƏMPVFGNWLQVGDDINHRNITDIAKKFGDI FLIRMGQRNIVVVSSPDIAKDV	90 90 90 90 90 90
AsC4H AcC4H PtC4H1 CaC4H GaC4H AtC4H	LHTQGVEFGSRTRNVVFDIFTGKGQDMVFTVYGEHWRKMRRIMTVPFFTNKVVNQYRMGWEDEAGRVVEDVKKDTKAAT3GIVLR&RLQL LHTQGVEFGSRTRNVVFDIFTGKGQDMVFTVYGEHWRKMRRIMTVPFFTNKVVNQYRMGWEDEAGRVVEDVKKDAKAAT3GIVLR&RLQL LHTQGVEFGSRTRNVVFDIFTGKGQDMVFTVYGEHWRKMRRIMTVPFFTNKVVQYRMGWEDEAAGVVEDVKKNPEAATAGIVLR&RLQL LHTQGVEFGSRTRNVVFDIFTGKGQDMVFTVYGEHWRKMRRIMTVPFFTNKVVQYRMGWEDEAASVVEDVKKNPEAATAGIVLR&RLQL LHTQGVEFGSRTRNVVFDIFTGKGQDMVFTVYGEHWRKMRRIMTVPFFTNKVVQYRHGWEDEAASVVEDVKKNPEAATAGIVLRRLQL LHTQGVEFGSRTRNVVFDIFTGKGQDMVFTVYGEHWRKMRRIMTVPFFTNKVVQYRHGWEDEAASVVEDVKKNPEAATAGIVLRRLQL LHTQGVEFGSRTRNVVFDIFTGKGQDMVFTVYGEHWRKMRRIMTVPFFTNKVVQYRHGWEDEAASVVEDVKKNPEAATAGIVLRKLQL	180 180 180 180 180 180
AsC4H AcC4H PtC4H1 CaC4H GaC4H AtC4H	MMYNNMYRIMFDRRFES <mark>GNDPLFLKLKAINNERSRLAQSFDYNYGDFIFILRPFLRGYLKIOKEVKERRIQLFKDYFLDERKKLSSTTIT MMYNNMYRIMFDRRFESENDPLFLKLKAINGERSRLAQSFDYNYGDFIFILRPFLRGYLKIOKEVKERRIQLFKDYFLDERKKLSSTKPT MMYNNMYRIMFDRRFESENDPLFNKLKAINGERSRLAQSFDYNYGDFIFILRPFLRGYLKIOKEVKERRIQLFKDYFVDERKKLSSTKNM MMYNNMFRIMFDRRFESENDPLFNKLKAINGERSRLAQSFDYNYGDFIFILRPFLRGYLKIOKEVKERRIQLFKDYFVDERKKLSSTKSM MMYNNMFRIMFDRRFESENDPLFNKLKAINGERSRLAQSFDYNYGDFIFILRPFLRGYLKIOKEVKERRIQLFKDYFVDERKKLSTKSM MMYNNMFRIMFDRRFESENDPLFVKLKAINGERSRLAQSFDYNYGDFIFILRPFLRGYLKIOKEVKERRIQLFKDYFVDERKKLSTKSM MMYNNMFRIMFDRRFESENDPLFVKLKAINGERSRLAQSFDYNYGDFIFILRPFLRGYLKION</mark>	270 270 270 270 270 270 270
AsC4H AcC4H PtC4H1 CaC4H GaC4H AtC4H	DNAGLKCAIDHIMDAEKKGEINEDNVLYIVENINVAAIETTLWSIEWGIAELVNHPEIQ <mark>RKLOHEI</mark> DTVLGP <mark>RPKITEPDIHRLPYE</mark> QAV DNAGLKCAIDHILDAEKKGEINEDNVLYIVENINVAAIETTLWSIEWGIAELVNHPKIQRKLOHEIDTVLGPGNQHTEPDHHRLPYLQAV NNAGLKCAIDHILDDOKKGEINEDNVLYIVENINVAAIETTLWSIEWGIAELVNHPEIQKKLRHEIDTULGPGQUTEPDHHRLPYLQAV NNAGLKCAIDHILDAOKKGEINEDNVLYIVENINVAAIETTLWSIEWGIAELVNHPEIQKKLRHEIDTULGPGQUTEPDHHRLPYLQAV NNAGLKCAIDHILDAOKKGEINEDNVLYIVENINVAAIETTLWSIEWGIAELVNHPEIQKKLRHEIDTULGPGQUTEPDHHRLPYLQAV SEGLKCAIDHILDAOKKGEINEDNVLYIVENINVAAIETTLWSIEWGIAELVNHPEIQKLRHEIDTULGPGQUTEPDHHRLPYLQAV	360 360 360 360 360 360
AsC4H AcC4H PtC4H1 CaC4H GaC4H AtC4H	VKETLRLRMAIPLLVPHMNL <mark>OD</mark> AKL <mark>SGN</mark> DIPAESKILVNAWWLANNPAHWKDPEEFRPERF <mark>D</mark> EEEAKVEVNGNDFRYTPFGVGRRSCFGI IKETLRLRMAIPLLVPHMNLHDAKLGGDIPAESKILVNAWWLANNPAHWKDPEEFRPERFDEEEAKVEANGNDFRYTPFGVGRRSCPGI IKETLRLRMAIPLLVPHMNLHDAKLGGDIPAESKILVNAWWLANNPAHWKNPEEFRPERFDEEEAKVEANGNDFRYTPFGVGRRSCPGI IKETLRLRMAIPLLVPHMNLHDAKLGGDIPAESKILVNAWWLANNPAHWKNPEEFRPERFDEEESKVEANGNDFRYTPFGVGRRSCPGI IKETLRLRMAIPLLVPHMNLHDAKLGGDIPAESKILVNAWWLANNPAHWKNPEEFRPERFDEESKVEANGNDFRYTPFGVGRRSCPGI IKETLRLRMAIPLLVPHMNLHDAKLGGDIPAESKILVNAWWLANNPAHWKNPEEFRPERFDEESKVEANGNDFRYTPFGVGRRSCPGI IKETLRLRMAIPLLVPHMNLHDAKLGGDIPAESKILVNAWWLANNPAHWKNPEEFRPERFDEESKVEANGNDFRYTPFGVGRRSCPGI VKETLRLRMAIPLLVPHMNLHDAKL	450 450 450 450 450 450
AsC4H AcC4H PtC4H1 CaC4H GaC4H AtC4H	ILALPILGITIGRIVQNFTLYPPFGMCKIDT EKGGQFSLHILNHETIVAKPRVF 505 ILALPILGITIGRIVQNFTLYPPFGMCKIDT EKGGQFSLHILNHSTIVAKPRVF 505 ILALPILGITIGRIVQNFTL PPFGQSKIDT EKGGQFSLHILNHSTIVAKPRSF 505 ILALPILGITIGRIVQNFTL PPFGQSKIDT EKGGQFSLHILNHSTIVAKPRVF 505 ILALPILGITIGRIVQNFTL PPFGQSKUDT EKGGQFSLHILNHSTIVAKPRVF 505	

Figure 4. Multiple sequence alignment of *A. sativum* C4H (AsC4H) and five other homologues in other plants: *A. cepa* AcC4H (AY541032), *P. trichocarpa* PtC4H1 (EU603304), *C. album* CaC4H (FJ821504), *G. arboretum* GaC4H (AF286648), and *A. thaliana* AtC4H (U37235). GenBank accession numbers are indicated in parentheses. Shaded areas indicate positions in the alignment with identical amino acids.



Figure 5. Phylogenic tree of AsC4H and some of its homologues: *A. cepa* AcC4H (AY541032), *P. trichocarpa* PtC4H1 (EU603304), *C. album* CaC4H (FJ821504), *G. arboretum* GaC4H (AF286648), *A. thaliana* AtC4H (U37235), and *O. sativa* OsC4H (AB207105). GenBank accession numbers are indicated in parentheses.



Figure 6. Relative expression levels of AsPAL and AsC4H in different organs of A. sativum. The expression level of the A. sativum actin gene was used as the reference. The values and error bars represent the mean  $\pm$  standard error from three samples.

**Expression Levels of the** *PAL* and *C4H* Genes in Different Organs of *A. sativum. AsPAL* was expressed in all of the tested organs (Figure 6). The highest expression level occurred in the root, where its relative expression (RQ value) to the actin gene was 40.79. Intermediate levels of expression were found in the bulbs, leaves, and scapes (RQ = 3.32, 4.17, and 23.14, respectively). The lowest expression level occurred in the bulbils (RQ = 1.64). *AsC4H* was expressed in the same pattern as *AsPAL*. Specifically, expression was the highest in the roots (RQ = 11.64), intermediate in the bulbs (RQ = 2.67), leaves

**Table 2.** Concentrations of Phenylpropanoid Compounds ( $\mu$ g/100 mg of DW) in Different Organs of Garlic<sup>a</sup>

compound	bulbils	scapes	leaves	bulbs	roots
4-hydroxybenzoic acid	0.23 (0.17)	0.02 (0.01)	0.06 (0.03)	$ND^b$	ND
chlorogenic acid	5.99 (0.63)	4.64 (0.54)	4.30 (0.17)	ND	1.55 (0.34)
caffeic acid	0.89 (0.13)	ND	0.82 (0.12)	ND	ND
<i>p</i> -coumaric acid	2.15 (0.01)	0.56 (0.04)	ND	0.03	0.44 (0.01)
ferulic acid	9.39 (0.30)	0.55 (0.04)	0.40 (0.05)	0.24 (0.06)	0.16 (0.00)
benzoic acid	ND	ND	0.65 (0.02)	Tr <sup>c</sup>	11.66 (1.47)
rutin	7.31 (0.35)	0.26 (0.05)	Tr	0.18 (0.02)	Tr
trans-cinnamic acid	0.09 (0.02)	ND	ND	0.02	ND
quercetin	35.23 (0.60)	ND	ND	ND	ND
kaempeferol	25.87 (0.56)	0.16 (0.03)	0.75 (0.07)	0.26 (0.04)	0.14 (0.02)

<sup>a</sup> Values represent the mean (SD) of three measurements. <sup>b</sup>ND = not detected. <sup>c</sup>Tr = trace amount (<0.001  $\mu$ g/100 mg of DW).

(RQ=2.11), and scapes (RQ=10.06), and lowest in the bulbils (RQ=0.55).

Analysis of Phenylpropanoid Compounds. 4-Hydroxybenzoic acid, chlorogenic acid, caffeic acid, p-coumaric acid, ferulic acid, benzoic acid, rutin, trans-cinnamic acid, quercetin, and kaempeferol were identified in different organs of A. sativum (Table 2). The concentrations of many of the identified compounds were very low or undetectable in the bulbs. The concentrations of these compounds, except (-)-catechin hydrate and benzoic acid, were higher in the bulbils than in other organs. For example, quercetin was only detected in the bulbils, where its concentration was  $35.23 \ \mu g/100 \ \text{mg}$  of dry weight (DW) (Table 2). Quercetin has been suggested as a potent anticancer agent in humans (26) and is also a strong antioxidant that can contribute to the prevention of atherosclerosis (27). Recently, quercetin has been shown to reduce the carcinogenic activity of several cooked food mutagens, enhance the antiproliferative activity of anticancer agents, and inhibit the growth of transformed tumorigenic cells (28). A high amount of kaempferol was detected in the bulbils (25.87  $\mu$ g/100 mg of DW), but a low amount was detected in scapes ( $0.16 \,\mu g/100$ mg of DW), leaves (0.75  $\mu$ g/100 mg of DW), bulbs (0.26  $\mu$ g/100 mg of DW), and roots (0.14  $\mu$ g/100 mg of DW) (Table 2). Currently, kaempferol has received much attention because of its antioxidant (29), antitumor, anti-inflammatory, and anti-ulcer activity (30) and its inhibitory activity of HIV protease (31).

We discovered that the bulbils, scapes, and leaves of garlic have higher concentrations of phenylpropanoid compounds than the bulb. In particular, the bulbils of garlic contain the highest concentrations of phenylpropanoids. However, the most commonly used part of garlic is the bulb. As a result, we need to raise consumer awareness of the quality and usefulness of other parts of garlic to maximize the health benefits of phenylpropanoids. For example, immature scapes have a mild taste and can be stirfried or eaten raw. Garlic leaves are also a popular vegetable in many parts of Asia.

This study is the first reported cloning and molecular characterization of AsPAL and AsC4H. Amino acid sequence alignments and phylogenetic analyses showed that AsPAL and AsC4H were highly homologous with their orthologues in other plants. The gene expression pattern of *AsPAL* was also very similar to that of *AsC4H*. Specifically, the expression of *AsPAL* and *AsC4H* in the roots and scapes were significantly higher than in other organs. The relatively high gene expression of *AsPAL* and *AsC4H* in the roots but not in the bulbils, where phenylpropanoid compounds are concentrated, suggests that a transport mechanism is involved. The accumulation of phenylpropanoid compounds is opposite the level of gene expression; this might be explained with the grafting experiment, which confirmed that flavonoids are capable of long-distance movement (*32*, *33*).

In conclusion, the sequence and expression analyses of AsPAL and AsC4H presented in this study establish the foundation for elucidating the phenylpropanoid biosynthesis pathway in *A*. *sativum* in more detail. Further research will lead to not only a better understanding of plant metabolism but also potential applications in biotechnology and medicine.

#### ABBREVIATIONS USED

DEPC, diethylpyrocarbonate; RACE, rapid amplification of cDNA ends; PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; HPLC, high-performance liquid chromatography; DW, dry weight.

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