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

6 **AUTHORS**, INCLUDING:

Pham anh Tuan
National Agriculture and Food Research Org…
31 PUBLICATIONS **114** CITATIONS

SEE PROFILE

Haeng-Hoon Kim
Sunchon National University
96 PUBLICATIONS **523** CITATIONS

SEE PROFILE

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

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

PHAM ANH TUAN,[†,‡] NAM IL PARK,[†,‡] XIAOHUA LI,[‡] HUI XU,[‡] HAENG HOON KIM,[§] AND SANG UN PARK*[,‡]

[‡]Department of Crop Science, College of Agriculture and Life Sciences, Chungnam National University, 220 Gung-Dong, Yuseong-Gu, Daejeon 305-764, Korea, and [§]National Agrobiodiversity Center, National Academy of Agricultural Science, Rural Development Administration (RDA), 249 Suin-Ro Kweonseonku, Suwon 441-857, Korea. [†] These authors contributed equally to this work.

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
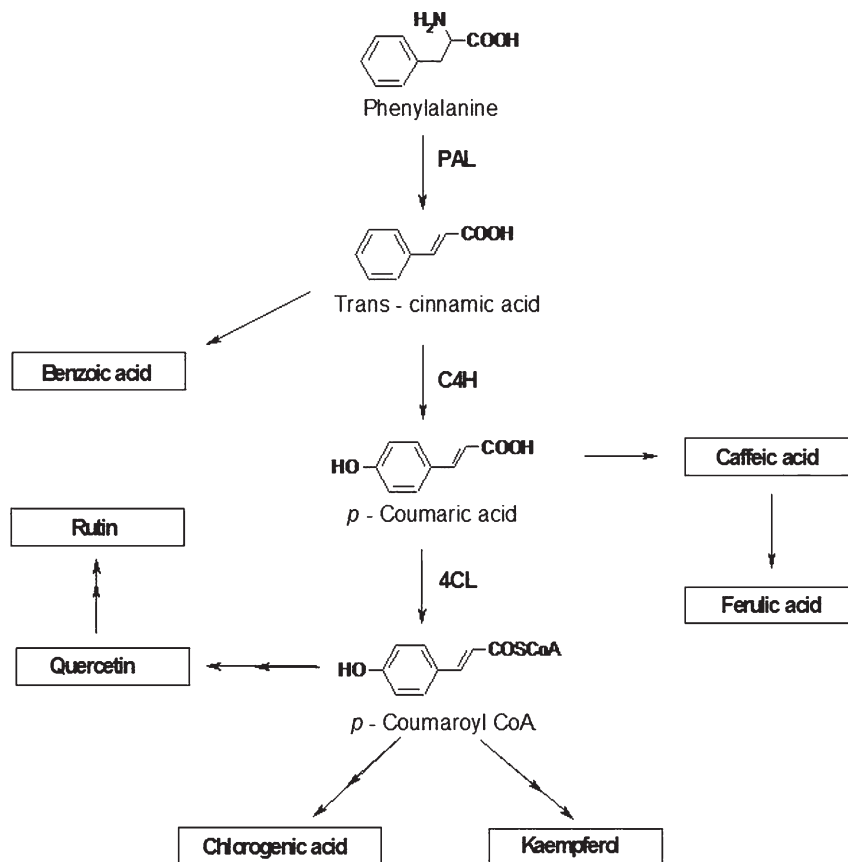
mechanical support, synthesis of anthocyanins, signaling via flavonoid nodulation factors, and protection against biotic and abiotic stresses (*14*−*16*). Research studies on PAL and C4H have important applications in biotechnology and elucidating the regulation of the phenylpropanoid biosynthesis pathway 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.

*To whom correspondence should be addressed: Department of Crop Science, College of Agriculture and Life Sciences, Chungnam National University, 220 Gung-Dong, Yuseong-Gu, Daejeon 305-764, Korea. Telephone: +82-42-821-5730. Fax: +82-42-822-2631. E-mail: supark@cnu.ac.kr.

10912 *J. Agric. Food Chem.,* Vol. 58, No. 20, 2010

Tuan et al.

Phenylalanine

|
PAL
↓

Trans - cinnamic acid

→ **Benzoic acid**

|
C4H
↓

p - Coumaric acid → **Caffeic acid**

Rutin ← → **Ferulic acid**

|
4CL
↓

Quercetin ← **p - Coumaroyl CoA**

↓ ↘

Chlorogenic acid   **Kaempferd**

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 μg of total RNA was reverse-transcribed using the ReverTra Ace-α 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 μL reaction volume with 0.4 μM 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 μm 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 μm, 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 μL. 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′—3′)	PCR product	vector for cloning
RACE PCR	conserved PAL_F	TMCARGGMTACTCHGGCATMMG		
	conserved PAL_R	GCGCTYTNSACRTGGTTNGTVA		
	conserved C4H_F	AACTGGCTBCARGTYGGVGAYGAY		
	conserved C4H_R	ACCANGCTRTVACMAGGATYTTG		
	GeneRacer_F	CGACTGGAGCACGAGGACACTGA	partial sequences of AsPAL or AsC4H	T-blunt
	GeneRacer_R	GCTGTCAACGATACGCTACGTAACG		
	PAL_F	GAAGTCGTTCACCAGCTCGGAGAAC		
	PAL_R	GACGTGTCAAGGAAAAAGCCGTG		
	C4H_F	CCATAGTGTCGTCTCGATCGCTGC		
	C4H_R	ATGAGAGGAGTAGGTTGGCGCAGAGTT		
real-time PCR	PAL_F	GCCTCACCCTTCGAGGTTCT		
	PAL_R	AGGTCTCCGCATCCTTCTCC		
	C4H_F	TTGGCCAAGAAGTTCGGAGA		
	C4H_R	TCCTGCCCTTTTCCAGTGAA		
	Actin_F	TGTTTCCTAGTATTGCTGGTAGA		
	Actin_R	AGCTCGTTGTAGAAAGTGTGAT		

```
AsPAL   ---------------MGAMNGDFSVNDEIIRIQDPLNWGAAAEPMSGSHLDEVKRMVKEYRGKSVKLEGATLKVGQVAAVAAG-EIKEVLDEGAREGVK 84
AcPAL   ---------------MGAVNGDFSVNNEIIRIDDPLNWGAAAKPMSGSHLDEVKRMVNEYREKSVKLDGATLKVAQVAAVAAG-EIKEVLDEGAREGVK 84
AtPAL1  MEINGAHKSNGGGVDAMLCGGDIKTKNMVINADDPLNWGAAAEQMKGSHLDEVKRMVAEFRKPVVNLGGESLTIGQVAAISTIGNSVKVELSELARAGVN 100
BrPAL1  MEVNG--LSHGGEVDAMLCGGEIK-KNATVVGADDPLNWGAAAEQMKGSHLDEVKRMVKEFRRPVVNLGGESLTIGQVAAISTLGNGVKVELSELARAGVK 97
PtPAL2  MEFCQ---------DSCTGNGSLG-----FNPNDPLNWGMVAESLKGSHLDEVKRMIDEMRKPVKLGGESLTIGQVTAIASRDVGVKVELSEPARVGVK 86

AsPAL   ASSDWVMDSMCKGTDSYGVTTGFGATSHRRTKNGAALQNELIRFLNAGIFGS-PNSGNSLESTTSRAAMLVRVNTLLQGYSGIRFEILESITRLNANIT 183
AcPAL   ASSDWVMDSMCKGTDSYGVTTGFGATSHRRTKNGVALQNELIRFLNAGIFGS-PNSGNSLESTTTRAAMLVRVNTLLQGYSGIRFEILESITRLNANIT 183
AtPAL1  ASSDWVMDSMNKGTDSYGVTTGFGATSHRRTKNGVALQKELIRFLNAGIFGSTKETSHTLPHSATRAAMLVRINTLLQGFSGIRFEILEAITSFLNNNIT 200
BrPAL1  ASSDWVMDSMGKGTDSYGVTTGFGATSHRRTKNGVALQKELIRFLNAGIFGSTKETSHTLPHSATRAAMLVRINTLLQGYSGIRFEILEAITSFLNNIT 197
PtPAL2  ASSDWVMDSMNKGTDSYGVTTGFGATSHRRTKCGGELQKELIRFLNAGIFGNGTESTHTLPHSASRAAMLVRINTLLQGYSGIRFEILEAITKLLNHNIT 186

AsPAL   PCLPLRGTITASGDLVPLSYIAALLTGRPNSKLVTSDNTLDTASEAFQLAGIDSGFFRLQPKEGLALVNGTAVGSGLASIVLYEINVLAVLAEVMSALFC 283
AcPAL   PCLPLRGTITASGDLVPLSYIAALLTGRPNSKSVTSDNTLDTPSEAFQLAGIDSGFFQLQPKEGLALVNGTAVGSGLASIVLYEINVLAVLAEVMSALFC 283
AtPAL1  PSLPLRGTITASGDLVPLSYIAGLLTGRPNSKATGPNGEALDAEAFKLAGISSGFFDLQPKEGLALVNGTAVGSGMASMVLFEANVLSVLAEILSAIFA 300
BrPAL1  PSLPLRGTITASGDLVPLSYIAGLLTGRPNSKATGPNGEALNADEAFKMAGVTSGFFDLQPKEGLALVNGTAVGSGMASMVLFEANVLSVLAEVMSAVFA 297
PtPAL2  PCLPLRGTITASGDLVPLSYIAGLLTGRPNSKAVGPNGETLAAPEAFTLAGINGGFFPLQPKEGLALVNGTAVGSGLASMVLFEANVIALLSEVISALFA 286

AsPAL   EVMCGKPEFTDHLTHKLKHHPGQIEVAAIMEHILDGSSYMKMAKNLHDMDPLQKPKQDRYALRTSPQWLGPQIEVIRAATKSIEREINSVNDNPLIDVSR 383
AcPAL   EVMCGKPEFTDHLTHKLKHHPGQIEAAAIMEHILDGSSYMKMAKLHDTDPLQKPKQDRYALRTSPQWLGPQIEVIRAATKSIEREINSVNDNPLIDVSR 383
AtPAL1  EVMSGKPEFTDHLTHRLKHHPGQIEAAAIMEHILDGSSYMKAAKKLHEMDPLQKPKQDRYALRTSPQWLGPQIEVIRYATKSIEREINSVNDNPLIDVSR 400
BrPAL1  EVMSGKPEFTDHLTHRLKHHPGQIEAAAIMEHILDGSSYMKAACKLHEMDPLQKPKQDRYALRTSPQWLGPQIEVIRAATKSIEREINSVNDNPLIDVSR 397
PtPAL2  EVMCGKPEFTDHLTHRLKHHPGQIEAAAIMEHILDGSSYVKAACKLHEIDPLQKPKQDRYALRTSPQWLGPLIEVIRTSTKMIERESINSVNDNPLIDVSR 386

AsPAL   NKAVHGGNFQGTPIGVSMDNTRLAVAAIGKLMFAQFSELVNDFYNNGLPSNLTGGRNPSLDYGFKGAEIAMASYCSELQFLANPVTNHVQSAEQHNQDVN 483
AcPAL   NKAVHGGNFQGTPIGVSMDNTRLAVAAIGKLMFAQFSELVNDFYNNGLPSNLTGGRNPSLDYGFKGAEIAMASYCSELQFLANPVTNHVQSAEQHNQDVN 483
AtPAL1  NKAIHGGNFQGTPIGVSMDNTRLAIAAIGKLMFAQFSELVNDFYNNGLPSNLTARNPSLDYGFKGAEIAMASYCSELQYLANPVTSHVQSAEQHNQDVN 500
BrPAL1  NKAIHGGNFQGTPIGVSMDNTRLAIAAIGKLMFAQFSELVNDYYNNGLPSNLTASRNPSLDYGFKGAEIAMASYCSELQFLANPVTSHVQSAEQHNQDVN 497
PtPAL2  NKAIHGGNFQGTPIGVSMDNTRLAIASIGKLMFAQFSELVNDYYNNGLPSNLTGGRNPSLDYGFKGAEIAMASYCSELQFLANPVTNHVQSAEQHNQDVN 486

AsPAL   SLGLISARKTDEAVDIILKLMSTTFLVALCQAVDLRHLEENLRQVVKGVVSQAVRKRVLTVGANGEPHPSRFCEKDLLKMIDREYVFTYADDACSAGYPLMQ 583
AcPAL   SLGLISARKTDEAVDIILKLMSTTFLVALCQAVDLRHLEENVRQAVKNAVSQAARRVLTVGANGEPHPSRFCEKDLLKMIDREYVFTYADDACSAAYPLMQ 583
AtPAL1  SLGLISSRKTSEAVDIILKLMSTTFLVALCQAVDLRHLEENLKQDVKNTVSQVAKKVLTIGVNGELHPSRFCEKDLLKVVDREQVFTYADDCSAIYPLIQ 600
BrPAL1  SLGLISSRKTSEAVDIILKLMSTTFLVGICQAVDLRHLEENLKQIVKNTVSQAAKKVLTIGVNGELHPSRFCEKDLLKVVDREQVFTYADDCSAIYPLIQ 597
PtPAL2  SLGLISARKTAEAVDIILKLMSTTWLVALCQAVDLRHIEENLKNTVKNTVSQAVKKRVLTMGFNGELHPSRFCEKDLLKVVDREHAFTYIDDCSAIYPLMQ 586

AsPAL   KVRQVIVDHALGNVMEREKDAETSIFQKIGAFEEELKRTLPKEVEAVRMAFDNGKCAVPNRIRECRSYPLYRLVREELGAYLTGEEGTSPGEVFDKVFEA 683
AcPAL   KVRQVIVDHALGNGEREKDSETSIFHKIGAFEEELKRTLPKEVEVRMAFDNGKCVIPNRIKECRSYPLYRLVREELGAYLAGEEGTSPGEVFDKVFEA 683
AtPAL1  KLRQVIVDHALINGESEKNAVTSIFHKIGAFEEELKAVLPKEVEAARAAYDNGTSAIPNRIKECRSYPLYRFVREELGTELLTGEKVTSPGEEFDKVFTA 700
BrPAL1  KLRQVIVDHALVNGESEKNAMTSIFHKIGAFEEELKAVLPKEVEAARAAYDNGTAAIPNRIKECRSYPLYRFVREELGTELLTGEKAISPGEEFDKVFTA 697
PtPAL2  KLRQVIVDHALMNGEKPHNSSTSIFQKIGAFEEELKTLLPKEVENARLELENGNPAIPNRITECRSYPLYKFVREELGTILLTGEKVGSPGEEFDKVETA 686

AsPAL   VCKGKVVDPLLECLQEWDGAPVPIC 708
AcPAL   VCNGKVVDPLLECLQEWDGAPLPIC 708
AtPAL1  ICPGKIIDPMMECLNEWNGAPIPIC 725
BrPAL1  ICPGKIIGPLMECLDEWNGAPIPIC 722
PtPAL2  ICAGKLIDPMLECLKEWNGAPIPIC 711
```
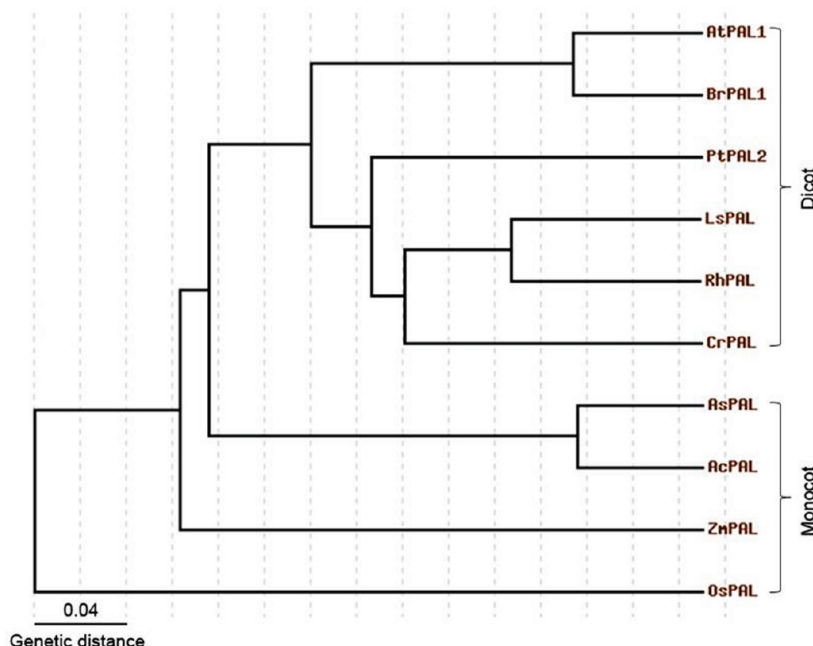
Figure 2. Multiple sequence alignment of *A. sativum* PAL (AsPAL) and four homologues in other plants: *A. cepa* AcPAL (AY541031), *A. thaliana* AtPAL1 (AY079363), *B. rapa* BrPAL1 (EU402423), and *P. trichocarpa* PtPAL2 (XM_002311977). GenBank accession numbers are indicated in parentheses. Shaded areas 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.

**10914** *J. Agric. Food Chem.,* Vol. 58, No. 20, 2010

Tuan et al.

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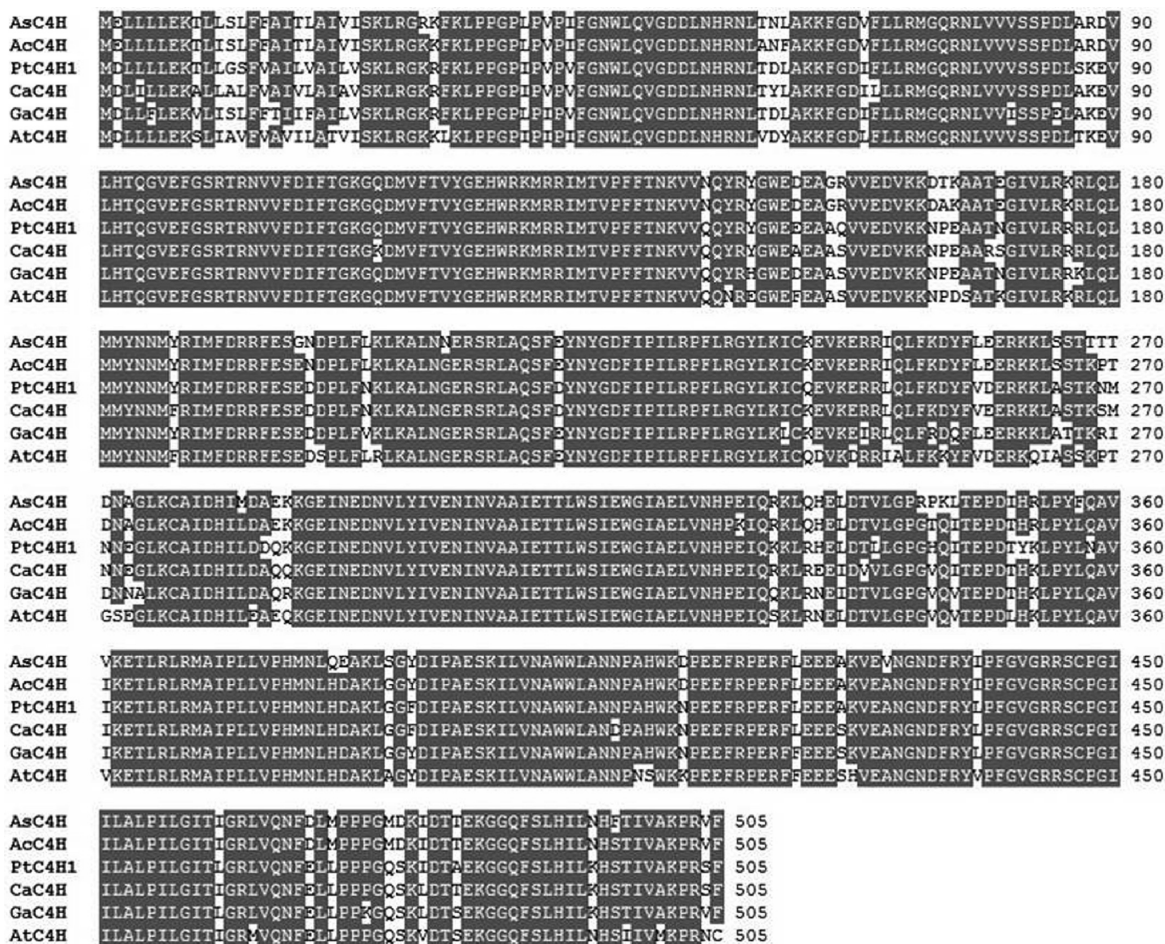
Figure 4. Multiple sequence alignment of *A. sativum* C4H (AsC4H) and five other homologues in other plants: *A. cepa* AcC4H (AY541032), *P. trichocarpa* PtC4H1 (EU603304), *C. album* CaC4H (FJ821504), *G. arboretum* GaC4H (AF286648), and *A. thaliana* AtC4H (U37235). GenBank accession numbers are indicated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

Article

J. Agric. Food Chem., Vol. 58, No. 20, 2010   **10915**
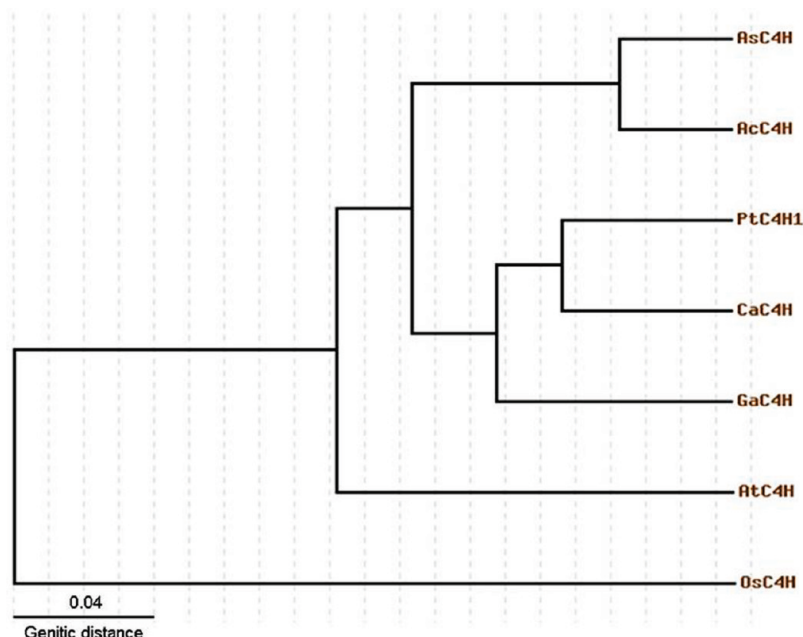
Figure 5. Phylogenic tree of AsC4H and some of its homologues: *A. cepa* AcC4H (AY541032), *P. trichocarpa* PtC4H1 (EU603304), *C. album* CaC4H (FJ821504), *G. arboretum* GaC4H (AF286648), *A. thaliana* AtC4H (U37235), and *O. sativa* OsC4H (AB207105). GenBank accession numbers are indicated in parentheses.
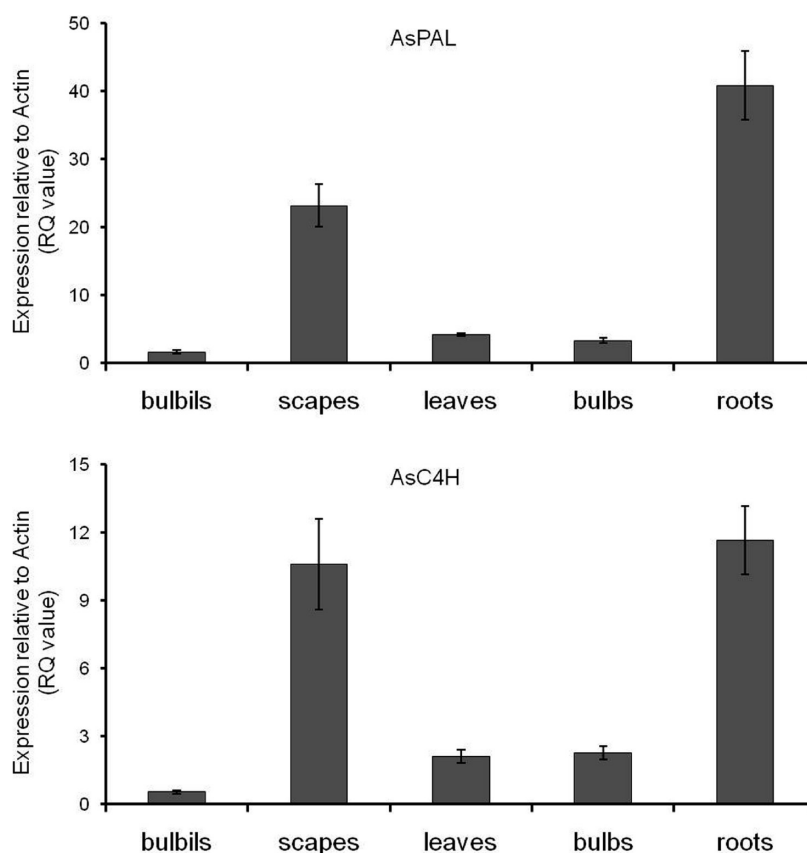
Figure 6. Relative expression levels of *AsPAL* and *AsC4H* in different organs of *A. sativum*. The expression level of the *A. sativum* actin gene was used as the reference. The values and error bars represent the mean ± standard error from three samples.

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

10916 *J. Agric. Food Chem.,* Vol. 58, No. 20, 2010

Tuan et al.

Table 2. Concentrations of Phenylpropanoid Compounds (μg/100 mg of DW) in Different Organs of Garlic[a]

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
p-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[c]	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
kaempferol	25.87 (0.56)	0.16 (0.03)	0.75 (0.07)	0.26 (0.04)	0.14 (0.02)

[a] Values represent the mean (SD) of three measurements. [b] ND = not detected. [c] Tr = trace amount (<0.001 μg/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 kaempferol 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 μg/100 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 μg/100 mg of DW), but a low amount was detected in scapes (0.16 μg/100 mg of DW), leaves (0.75 μg/100 mg of DW), bulbs (0.26 μg/100 mg of DW), and roots (0.14 μg/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 stir-fried 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.

LITERATURE CITED

(1) Tapsell, L. C.; Hemphill, I.; Cobiac, L.; Patch, C. S.; Sullivan, D. R.; Fenech, M.; Roodenrys, S.; Keogh, J. B.; Clifton, P. M.; Williams, P. G.; Fazio, V. A.; Inge, K. E. Health benefits of herbs and spices: The past, the present, the future. *Med. J. Aust.* **2006**, *185* (4), S1−S24.

(2) Rivlin, R. S. Historical perspective on the use of garlic. *J. Nutr.* **2001**, *131* (3), 951S−954S.

(3) Rivlin, R. S. Is garlic alternative medicine? *J. Nutr.* **2006**, *136* (3), 713S−715S.

(4) Pittler, M. H.; Ernst, E. Clinical effectiveness of garlic (*Allium sativum*). *Mol. Nutr. Food Res.* **2007**, *51* (11), 1382−1385.

(5) Butt, M. S.; Sultan, M. T.; Butt, M. S.; Iqbal, J. Garlic: Nature's protection against physiological threats. *Crit. Rev. Food Sci. Nutr.* **2009**, *49* (6), 538−551.

(6) Iciek, M.; Kwiecien, I.; Wodek, L. Biological properties of garlic and garlic-derived organosulfur compounds. *Environ. Mol. Mutagen.* **2009**, *50* (3), 247−265.

(7) Swiderski, F.; Dabrowska, M.; Rusaczonek, A.; Waszkiewicz-Robak, B. Bioactive substances of garlic and their role in dietoprophylaxis and dietotherapy. *Rocz. Panstw. Zakl. Hig.* **2007**, *58*, 41−46.

(8) Benkeblia, N. Antimicrobial activity of essential oil extracts of various onions (*Allium cepa*) and garlic (*Allium sativum*). *Lebensm.-Wiss. Technol.* **2004**, *37* (2), 263−268.

(9) Dixon, R. A.; Paiva, N. L. Stress-induced phenylpropanoid metabolism. *Plant Cell* **1995**, *7*, 1085−1097.

(10) Barber, M. S.; Mitchell, H. J. Regulation of phenylpropanoid metabolism in relation to lignin biosynthesis in plants. *Int. Rev. Cytol.* **1997**, *172*, 243−293.

(11) Harakava, R. Genes encoding enzymes of the lignin biosynthesis pathway in Eucalyptus. *Genet. Mol. Biol.* **2005**, *28*, 601−607.

(12) Liu, R.; Xu, S.; Li, J.; Hu, Y.; Lin, Z. Expression profile of a PAL gene from *Astragalus membranaceus* var. *Mongholicus* and its crucial role in flux into flavonoid biosynthesis. *Plant Cell Rep.* **2006**, *25* (7), 705−710.

(13) Russel, D. W. The metabolism of aromatic compounds in higher plants. X. Properties of the cinnamic acid 4-hydroxylase of pea seedlings and some aspects of its metabolic and developmental control. *J. Biol. Chem.* **1971**, *246*, 3870−3878.

(14) Whetten, R. W.; Sederoff, R. R. Phenylalanine ammonia-lyase from loblolly pine: Purification of the enzyme and isolation of complementary DNA clones. *Plant Physiol.* **1992**, *98* (1), 380−386.

(15) Holton, T. A.; Cornish, E. C. Genetics and biochemistry of anthocyanin biosynthesis. *Plant Cell* **1995**, *7* (7), 1071−1083.

Article

J. Agric. Food Chem., Vol. 58, No. 20, 2010   **10917**

(16) Weisshaar, B.; Jenkins, G. I. Phenylpropanoid biosynthesis and its regulation. *Curr. Opin. Plant Biol.* **1998**, *1* (3), 251−257.

(17) Singh, K.; Kumar, S.; Rani, A.; Gulati, A.; Ahuja, P. Phenylalanine ammonia-lyase (PAL) and cinnamate 4-hydroxylase (C4H) and catechins (flavan-3-ols) accumulation in tea. *Funct. Integr. Genomics* **2009**, *9* (1), 125−134.

(18) Teutsch, H. G.; Hasenfratz, M. P.; Lesot, A.; Stoltz, C.; Garnier, J. M.; Jeltsch, J. M.; Durst, F.; Werch-Reichhart, D. Isolation and sequence of a cDNA encoding the Jerusalem artichoke cinnamate-4-hydroxylase, a major plant cytochrome P450 involved in the general phenylpropanoid pathway. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 4102−4106.

(19) Song, J.; Wang, Z. Molecular cloning, expression and characterization of a phenylalanine ammonia-lyase gene (*SmPAL1*) from *Salvia miltiorrhiza*. *Mol. Biol. Rep.* **2009**, *36* (5), 939−952.

(20) Okada, T.; Mikage, M.; Sekita, S. Molecular characterization of the phenylalanine ammonia-lyase from *Ephedra sinica*. *Biol. Pharm. Bull.* **2008**, *31* (12), 2194−2199.

(21) Wang, Y.; Chen, J.-Y.; Jiang, Y.-M.; Lu, W.-J. Cloning and expression analysis of phenylalanine ammonia-lyase in relation to chilling tolerance in harvested banana fruit. *Postharvest Biol. Technol.* **2007**, *44* (1), 34−41.

(22) Kervinen, T.; Peltonen, S.; Utriainen, M.; Kangasjärvi, J.; Teeri, T. H.; Karjalainen, R. Cloning and characterization of cDNA clones encoding phenylalanine ammonia-lyase in barley. *Plant Sci.* **1997**, *123* (1−2), 143−150.

(23) Bozin, B.; Mimica-Dukic, N.; Samojlik, I.; Goran, A.; Igic, R. Phenolics as antioxidants in garlic (*Allium sativum* L., Alliaceae). *Food Chem.* **2008**, *111* (4), 925−929.

(24) Cramer, C.; Edwards, K.; Dron, M.; Liang, X.; Dildine, S.; Bolwell, G. P.; Dixon, R.; Lamb, C.; Schuch, W. Phenylalanine ammonia-lyase gene organization and structure. *Plant Mol. Biol.* **1989**, *12*, 367−383.

(25) Schuster, B.; Retey, J. Serine-202 is the putative precursor of the active site dehydroalanine of phenylalanine ammonia lyase. Site-directed mutagenesis studies on the enzyme from parsley (*Petroselinum crispum* L.). *FEBS Lett.* **1994**, *349*, 252−254.

(26) Yoshida, M.; Sakai, T.; Hosokawa, N.; Marui, N.; Matsumoto, K.; Fujioka, A.; Nishino, H.; Aoike, A. The effect of quercetin on cell cycle progression and growth of human gastric cancer cells. *FEBS Lett.* **1990**, *260* (1), 10−13.

(27) Hollman, P. C. H.; van Trijp, J. M. P.; Buysman, M. N. C. P.; van der Gaag, M. S.; Mengelers, M. J. B.; de Vries, J. H. M.; Katan, M. B. Relative bioavailability of the antioxidant flavonoid quercetin from various foods in man. *FEBS Lett.* **1997**, *418* (1−2), 152−156.

(28) Leighton, T.; Ginther, C.; Fluss, L.; Harter, W. K.; Cansado, J.; Notario, V. Quercetin and its glycosides in *Allium* vegetables. In *Phenolic Compounds in Food and Their Effects on Health II*; Ho, C.-T., Lee, C. Y., Huang, M.-T., Eds.; American Chemical Society (ACS): Washington, D.C., 1992; ACS Symposium Series, Vol. 507, Chapter 16, pp 220−238.

(29) Vinson, J. A.; Dabbagh, Y. A.; Serry, M. M.; Jang, J. Plant flavonoids, especially tea flavonols, are powerful antioxidants using an in vitro oxidation model for heart disease. *J. Agric. Food Chem.* **1995**, *43* (11), 2800−2802.

(30) Goel, R. K.; Pandey, V. B.; Dwivedi, S. P.; Rao, Y. V. Anti-inflammatory and antiulcer effects of kaempferol, a flavone, isolated from *Rhamnus procumbens*. *Indian J. Exp. Biol.* **1988**, *26* (2), 121−124.

(31) Brinkworth, R. I.; Stoermer, M. J.; Fairlie, D. P. Flavones are inhibitors of HIV-1 proteinase. *Biochem. Biophys. Res. Commun.* **1992**, *188* (2), 631−637.

(32) Buer, C. S.; Muday, G. K.; Djordjevic, M. A. Flavonoids are differentially taken up and transported long distances in *Arabidopsis*. *Plant Physiol.* **2007**, *145* (2), 478−490.

(33) Buer, C. S.; Muday, G. K.; Djordjevic, M. A. Implications of long-distance flavonoid movement in *Arabidopsis thaliana*. *Plant Signaling Behav.* **2008**, *3* (6), 415−417.