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## **Research** Note

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# Molecular cloning of 4-coumarate: CoA ligase and total phenolic content in garlic (*Allium sativum*)

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#### Abstract

*Allium sativum* L. belongs to a member of the onion family (Alliaceae) and has been used for both culinary and medical purpose. We cloned 4-coumarate:CoA ligase (4CL) from *Allium sativum*. 4-Coumarate:CoA ligase has an important role in the biosynthesis of plant secondary metabolites at the divergence point from general phenylpropanoid metabolism to several major branch pathways. Its deduced amino acid sequence was 69–82% identical to its orthologs in other plants. The expression level of *As4CL* was the highest in the roots and the lowest in bulbils. In addition, phenolic compounds were abundant in the leaves but not in bulbs, which are the most commonly used part of garlic.

**Key words:** *Allium sativum*; garlic; phenolic content; 4-coumarate:CoA ligase. **Abbreviation:** PAL-phenylalanine ammonia-lyase; C4H-cinnamate 4-hydroxylase; 4CL-4-coumarate:CoA ligase; RACE-rapid amplification of cDNA ends; GAE-gallic acid equivalent; BLAST-Basic Local Alignment Search Tool.

#### Introduction

In many plants, phenolic compounds are natural products that contribute to the color of plants, play an essential role in their reproduction and growth, and protect against pathogens, parasites, and predators (Báidez et al., 2007). In addition, many phenolic compounds have potent pharmcological properties, such as antioxidant, anticancer, antiatherosclerotic, antibacterial, antiviral, and anti-inflammatory effects (Benavente-Garc et al., 2000; Han et al., 2007; Manach et al., 2005; Owen et al., 2000). Phenolic compounds, such as flavonoids, stilbenes, coumarins, suberin, and lignin, are mostly synthesized from the phenylpropanoid pathway. There are 3 core reactions in this pathway: (1) phenylalanine ammonia-lyase (PAL) catalyzes the deamination of phenylalanine to produce trans-cinnamic acid, (2) cinnamate 4-hydroxylase (C4H) converts trans-cinnamic acid to pcoumaric acid, and (3) 4-coumarate:CoA ligase (4CL) uses pcoumaric acid to synthesize p-coumaroyl CoA. The gene expression of 4CL, like that of many phenylpropanoid enzymes, is stimulated by pathogens, wounding, and ultraviolet (UV) irradiation (Douglas et al., 1991; Ellard-Ivey and Douglas, 1996; Uhlmann and Ebel, 1993). In Populus tremuloides, the suppression of 4CL reduces lignin biosynthesis by 45% (Hu et al., 1999). A similar reduction in the lignin biosynthesis occurs in transgenic tobacco with decreased 4CL activity (Kajita et al., 1997). Garlic (Allium sativum L.) is a widely cultivated plant that has sulfurcontaining and phenolic compounds, which have antimicrobial, antifungal, anti-inflammatory, antioxidant, antitumor, and cardioprotective properties (Bhagyalakshmi et al., 2005; Bozin et al., 2008). In this study, we cloned and

characterized 4CL for the first time in garlic. In addition, we determined the total phenolic content in different organs.

#### Materials and methods

#### Plant Material

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

#### Isolation of cDNA encoding As4CL

For cloning, the total RNA was extracted from 100 mg of each powdered organ using the Plant Total RNA Mini Kit (Geneaid, Sijhih, Taiwan). Then, the total RNA was used to synthesize first-strand cDNA using the GeneRacer Kit (Invitrogen, Carlsbad, CA). We used degenerate forward (5'-ARCARGTNGAYGGNGAVAAYCCBA-3') and reverse (5'-ASCCATTRWATTTGATVADYTCCT-3') polymerase chain reaction (PCR) primers to obtain a fragment of A. sativum 4CL (As4CL). These degenerate primers were designed to match a conserved region within 4CL. Subsequently, we isolated the full-length As4CL by using 5' and 3' rapid amplification of cDNA ends (RACE) PCR with specific primers, namely, As4CL\_3' (5'-GCTAAGGAGCCTTTTGATGTGAAAT-3') and As4CL\_5'

	Y	
As4CL	MGSISMHQETIFRSKLPDIYIPLHLPLHSYCFENIQEFSDKPCTICGTTEKVYTYSDVELTSKRVAVGLHELGIRKCDVIMILLP	85
Ac4CL	MGSISMDQETIFRSKLPDIYIPHLPLHSYCFQHIQEFSDKPCTITGITEKVYTYADVELTSKRVAVGLRDLGIRKGHVIMILLP MFMETTTETKQSGDIIFRSKLPDIYIPKHLPLHSYCFENISEFSSRPCTIXGANDQIYIYADVELTCRKVAVGLNKLGIQQKDIMILLP	85
Nt4CL1	MEMETTTETKQSGDLIFRSKLPDIYIPKHLPLHSYCFENISEFSSRPCLISGANDQIYTYAPVELTCRKVAVGLNKLGIQQKDTIMILLP	90
Ca4CL2	MPMENETRDLIFRSKLPDIYIPWHLPLHSYCFENISEFNSRPCLIDGANDQIYSYAPVELTSRKVAVGLNKLGVQQKDDIMILLP	85
Pf4CL	MEAENDQAQEFIFRSKLPDIHIENHLPLHTYCFENLSREKDNPCLINGPTGEIHTYADVELTSRKVASGLNKLGIKQGDVILLLLQ	86
As4CL	NCPEFAYSFIGASYLGAMSTANPYYTPAEIKKQALCSSVRVMIIZSCYVEKIRD-LENN-VKIIVIDESVDGHSACIPFSQSSSADER	
Ac4CL	NSPEFAFSFLGASYLGAMSTTANPYYTFAEIKKQAMCSGVRVIITESCYVFKIKDLEHN-VKIVVIDELVDEHSTCIFFSCLSSADER	
Nt4CL1	NSPEFVFAFMGASYLGAISTVANPLFTFAEVVKQAKASSAKIIITQSCFVCKVKDYASEND-VKVICIDSAPEGCLHFSDLTQSDEH	
Ca4CL2	NSPEFYFAFMGASYLGAISTYANPLFTFAEVIKQAKASSAKIIITLACYICKVKDYATEND-VKLICIDSAPEGCIHFSDLTQSDEH	171
Pf4CL	NSPEFVFAFLGASIIGAISTIANPFYTPAEVAKQATASKAKLIITQAVYABKVQQFVKENDHVKIVTVDSPPENYLHESPLINSDED	173
	V V	0.00
As4CL	NLP <sup>EVD</sup> ISPDDVVALPYSSGTTGLPKGVMLTHEGL <mark>I</mark> TSVAQQVDGENENLYFRSDDVPLCVLPLFHIYSLNSVLLCGLRAGSTILLVKKF KLPEVEISPDDVVALPYSSGTTGLPKGVMLTHEGLITSVAQQVDGENENLYFRSDDVLLCVLPLFHIYSLNSVLLCGLRAGSTILLMRKF	262
Ac4CL	EIPDVEISPDDVVALPYSSGTIGLPKGVALIHEGLTISVAQQVDGENANLYHESDDVLLCVLPLFHIISLNSVLLCGLRAGSTIDDMRKF	262
Nt4CL1		
Ca4CL2 Pf4CL	EIPIVAICPDDVVALFYSSGTTGLPKGVMLTHKGLVTSVAQQVDGENANLY <mark>HESP</mark> DVLMCCLPLFHIYSLNSVLLCGLRIGAAILIMQKF	261
PI4CL	DIPAVEINPDDVVALPYSSGTTGLPKGVMLTHKGLVTSVAQQVDGENENLYFHEKDVIICVLPLFHIYSLNSVLLCGLRVGSAIL	263
As4CL	Box I DISKVVELIGKYEVTIAPFVPPICVEIAKNDVVGMCNLSNIR VMSGAAPYGKELEDRIKCKYPNAVLGQGYGMTEAGPVLSMCLAFAKE	352
Ac4CL	DLTKVVELNGKI VIIAPIVETICIPICIPICIPICIPICAPIACULINIR VMSGAAPIGKOLEDKUKKI MANGOOGIGMLEKGPVL GOLAFAKE	352
Nt4CL1	DIAPFIELIÇKYKVSIGPFVPPIVLAIAKSPIVDSYDLSSVRIVMSGAAPIGKELEDAVRIKFPNAKIGÇGYGMTEAGPVLAMCLAFAKE	356
Ca4CL2	DIVHFIELICKYKVTIGPFVPPIVLAIAKSPIVDSDEDSVKTVBSGAAPIGKELEDIVRIKFRANCGGGGGMEAGPVLANCLAFAKE	351
Pf4CL	EIVTLMELVQKYKVTIAPFVPPVVLAVAKCPVVDKYDLSSIRTVMSGAAPMGKELEDTVRAKLPNAKLGQGYGMTEAGPVLSMCLAFAKE	353
	The second se	
As4CL	PFDVKSGSCGTVVRNAELKIVDPDTGISLSKNQPGEICIRGXQIMKGYLNDLEATERTIDXEGWLHTGDIGYVDXDDELFIVDRLKELVK	442
Ac4CL	PTQAKSGSCGT VRNAELKVYDP TG SLCRN PSEICIRG QIMKGYLND EATSSTID EGWLHTGDIGYVD DDEVFIVDR KELIK	442
Nt4CL1	PF <mark>DI</mark> KSG <sup>2</sup> CGTVVRNAE <mark>4</mark> KIVDPDTG <mark>2</mark> SL <mark>F</mark> RNQFGEICIRG <sup>2</sup> QIMKGYLND <mark>F</mark> EAT <mark>I</mark> RTID <mark>K</mark> EGWLHTGDIG <mark>FID</mark> 2DDE <mark>F</mark> FIVDRLKELIK	446
Ca4CL2	PFEIKSGACGTVVRNAEMKIVDPDTG <mark>C</mark> SLERNQFGEICIRGEQIMKGYLND <mark>F</mark> ESTERTID <mark>X</mark> EGWLHTGDMGFID <mark>NDGEL</mark> FIVDRLKELIK	441
Pf4CL	PFFIKSGACGTVVRNAEMKIVDPDTGRSLFRNQAGEICIRGSQIMKGYLND <mark>F</mark> EATERT <mark>NDND</mark> GWLHTGDIG <mark>YID</mark> GDDE <mark>I</mark> FIVDRLKELIK	443
	Box IIBox II	
As4CL	YKGFQVAFAE <mark>TEAMFIAHPEVAG</mark> AAVV <mark>S</mark> MKDEVAGETPVAFVVR <mark>S</mark> NGSDITEDE <mark>TKKY</mark> ISKQV <mark>T</mark> FYRR <mark>THK</mark> VFFVDSIPKAPSGKILRKE	532
Ac4CL	EKGEQVEPAEIESTIVSHPOIDAAVIPOKOEVAGEVPVAFVVKASGSDITEDAVKEFISKQVVFYKRLQIVYFYHAIPKSPSGKILKKD	532
Nt4CL1	YKGFQVAFAE <mark>HEAT</mark> L <mark>INHEN</mark> IS <mark>DAAVVEMKDEQ</mark> AGEVEVAFVVRSNGSAITEDE <mark>VKDF</mark> ISKQV <mark>I</mark> FYKR <mark>VKR</mark> VFFV <mark>ETV</mark> FKSESGKILRKD	536
Ca4CL2	YKGFQVAFAELEALLNHENIS <mark>DAAVVFMKDEQ</mark> AGEVFVAFVVRSNGSTITEDEVK <mark>DFV</mark> SKQV <mark>V</mark> FYKR <mark>IKR</mark> VFFV <mark>ETV</mark> FKSFSGKILRKD	531
Pf4CL	YKGFQVAFAE <mark>leayliahed</mark> is <mark>doavvpmkdea</mark> agevp <mark>i</mark> afvvrangskitede <mark>ikqy</mark> iskqv <mark>v</mark> fykr <mark>isr</mark> vff <mark>teai</mark> fkapsgkilrkd	533
As4CL	ERALERAG-YPDVQ 545	
AC4CL	LRARLSSFT 541	
Nt4CL1	LRARLANG-VPN 547	
Ca4CL2	LRARLANG-VIN 542	
Pf4CL	LRARLANGDLPH 545	

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Fig. 1. Multiple sequence alignment of the deduced amino acid sequence of As4CL and orthologous 4CL sequences from other plants. Boxes I and II highlight 2 highly conserved motifs (see text for details). The arrows indicate conserved cysteine residues. Ac4CL (GenBank accession no. AY541033) from *Allium cepa*, Nt4CL1 (U50845) from *Nicotiana tabacum*, Ca4CL2 (EU616540) from *Capsicum annuum*, Pf4CL (FJ230968) from *Paulownia fortunei*.

(5'-CCTCCAGATCGTTCAGATATCCTTT-3'). Finally, the PCR products were purified and cloned into a T-blunt vector (SolGent, Daejeon, Koprea) and sequenced by the National Instrumentation Center for Environmental Management (NICEM, Seoul National University, Korea).

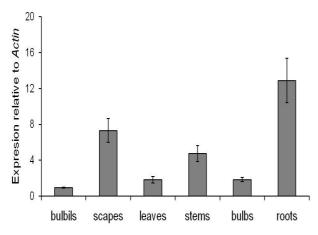
#### Quantitative Real-time PCR

For gene expression analysis, we synthesized cDNA from equal concentrations of total RNA from different organs of A. sativum by using the ReverTra Ace-a- kit (Toyobo, Osaka, Japan). Quantitative Real-time PCR was performed with the primers As4CL\_F (5'-AGGATGGTTGCATACAGGAGACA-3') and As4CL\_R (5'-CTCTCCAGCCACTTCATCTTTCA-3') by using the SYBR Green Realtime PCR Master Mix kit (Toyobo). We AsACTIN\_F (5'also used the primers TGTTTCCTAGTATTGCTGGTAGA-3') and AsACTIN\_R (5'-AGCTCGTTGTAGAAAGTGTGAT-3') to amplify the A. sativum actin gene (GenBank accession number: AY821677), which served as an internal reference. The real-time PCR reaction products were analyzed by using MJ Opticon

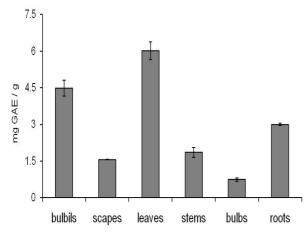
Monitor software (BioRad, Hercules, CA). All experiments were performed in triplicate.

#### Total phenolic contents from garlic

To extract the phenolic compounds from A. sativum, a powdered sample (0.2 g) was incubated in 5 mL of 80% methanol in an ultrasonic bath at 40 °C for 30 min. Then, the extracts were centrifuged at 920 ×g for 10 min. The total phenolic content of the extracts was determined by using the Folin-Ciocalteu method with some modifications (Singleton et al., 1999). Briefly, 100 µL of the extracts or a standard solution of gallic acid  $(0.5, 1, 1.5, 2, 2.5, and 3 \text{ mg} \cdot \text{mL}^{-1})$  was add to 1.5 mL of distilled deionized water (DDW), and then mixed with 100 µL of 2 N Folin-Ciocalteau reagent (Sigma, St Louis, Mo). After 5 min, the reaction was neutralized with 1.5 mL of 7.5%  $Na_2CO_3,$  and then incubated at 30  $^{\circ}\!C$  for 90 min. Subsequently, the absorbance of the sample was measured spectrophotometrically at 760 nm. The total phenolic content was calculated from a standard curve and expressed as mg gallic acid equivalent (GAE) ·g<sup>-1</sup> dry weight. All measurements were performed in triplicate.



**Fig** 2. Expression levels of *As4CL* mRNA transcripts relative to that of actin in different organs of *A. sativum*. The values and error bars indicate the mean and standard error, respectively, from 3 independent measurements.



**Fig 3.** Total content of phenolic compounds in extracts of different organs of *A. sativum*. The values and the error bars indicate the mean and standard error, respectively, from 3 independent measurements. GAE; gallic acid equivalent.

#### **Results and discussion**

The open reading frame of As4CL (GenBank accession number HQ171898) was 1635 nucleotides long and encoded a 545 amino acid protein. A Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST) analysis showed that As4CL shares 82% identity and 92% similarity with A. cepa 4CL, 70% identity and 86% similarity with N. tabacum 4CL, 69% identity and 86% similarity with C. annuum 4CL, and 69% identity and 85% similarity with P. fortunei 4CL (Fig. 1). As4CL also contained 2 highly conserved motifs for an AMP-binding domain (Allina et al., 1998; Becker-André et al., 1991) and the 4CL catalytic site (Ehlting et al., 1999) (Fig. 1, Boxes 1 and 2). In addition, 6 conserved cysteine residues were identified (Fig. 1) (Ehlting et al., 2001). As4CL was constitutively expressed in all of the organs that we examined (Fig. 2). The relative expression level of As4CL to that of the actin gene in the roots (RQ = 12.9) was higher than that in the scapes (RQ = 7.32), stems (RQ = 4.74), bulbs (RQ = 1.85), or leaves (RQ = 1.81). However, it was barely expressed in bulbils (RQ = 0.94). Although many parts of the garlic plant are used for various purposes, the bulbs are the most frequently used part (Nasim et al., 2009). As shown in Fig. 3, the total phenolic content in different organs of garlic ranged from 0.75 to 6.01 mg GAE/g. Phenolic compounds were more abundant in the leaves and bulbils (6.01 and 4.48 mg GAE·g<sup>-1</sup>, respectively) than in the roots (2.99 mg GAE·g<sup>-1</sup>), scapes (1.57 mg GAE·g<sup>-1</sup>), or stems (1.86 mg  $GAE \cdot g^{-1}$ ). The concentration of phenolic compounds was the lowest in the bulbs (0.75 mg GAE $\cdot$ g<sup>-1</sup>). The expression pattern of As4CL was similar to those of AsPAL and AsC4H (Tuan et al., unpublished results), which is consistent with the presence of common *cis*-elements in the promoter region of PAL, C4H, and 4CL in several plants (Bell-Lelong et al., 1997; Logemann et al., 1995). Furthermore, the high expression level of 4CL in the roots of A. sativum is in agreement with the importance of 4CL in the production of lignin (Hu, et al., 1999; Lee et al., 1997; Li et al., 2003) and the high rate of lignification during root development (Dixon et al., 1994). In addition, the moderately high content of phenolic compounds in the roots suggested that As4CL regulates the flux of lignin synthesis in the roots. In contrast, the content of phenolic compounds in the bulbs was very low, despite their strong flavor, which is due to sulfur-containing compounds (Milner, 2001). A similar inverse relationship between the concentrations of sulfurcontaining compounds and a phenolic compound, flavonol, has been reported in garlic and onion (Park et al., 2008). As a result, the low content of phenolic compounds in garlic bulbs might be due to the high content of sulfur-containing compounds in these organs. Further studies are needed to elucidate the relationships between the accumulation of phenolic compounds and the expression of biosynthetic genes in A. sativum. Currently, we are cloning more genes that are involved in the synthesis of phenolic compounds.

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