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Research Note

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, anti-atherosclerotic, 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 p-coumaric acid, and (3) 4-coumarate:CoA ligase (4CL) uses p-coumaric 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 sulfur-containing 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′

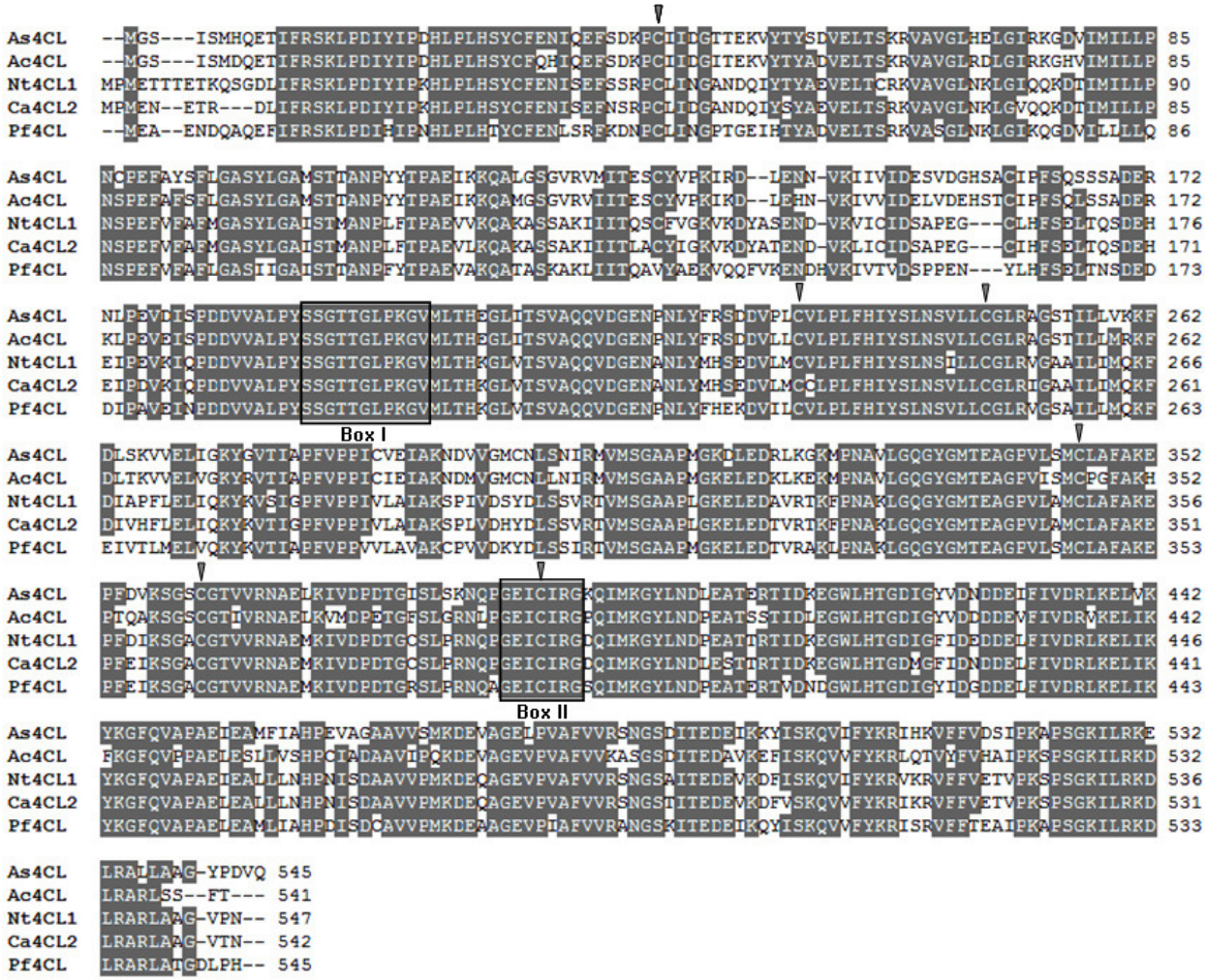

Fig. 1. Multiple sequence alignment of the deduced amino acid sequence of As4CL and orthologous 4CL sequences from other plants. Boxes I and II highlight 2 highly conserved motifs (see text for details). The arrows indicate conserved cysteine residues. Ac4CL (GenBank accession no. AY541033) from *Allium 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-α- 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 also used the primers AsACTIN_F (5′-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 ℃ 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 mg·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 ℃ 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^{-1} dry weight. All measurements were performed in triplicate.

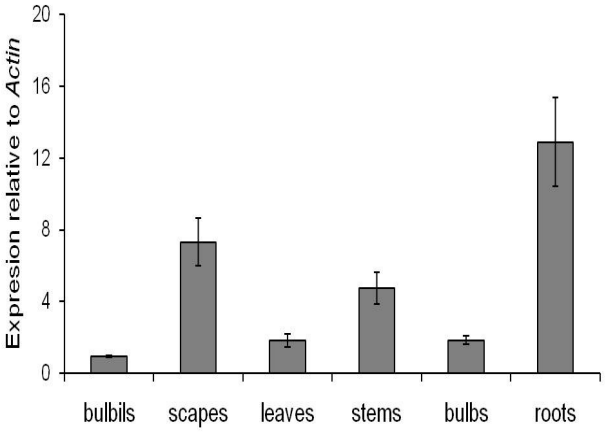

Fig 2. Expression levels of *As4CL* mRNA transcripts relative to that of actin in different organs of *A. sativum.* The values and error bars indicate the mean and standard error, respectively, from 3 independent measurements.

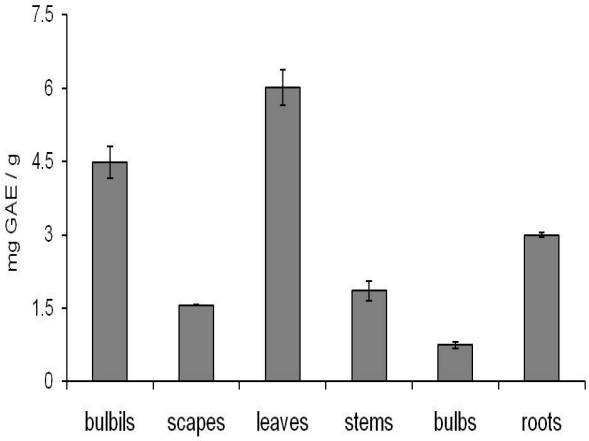

Fig 3. Total content of phenolic compounds in extracts of different organs of *A. sativum*. The values and the error bars indicate the mean and standard error, respectively, from 3 independent measurements. GAE; gallic acid equivalent.

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^{-1}, respectively) than in the roots (2.99 mg GAE·g^{-1}), scapes (1.57 mg GAE·g^{-1}), or stems (1.86 mg GAE·g^{-1}). The concentration of phenolic compounds was the lowest in the bulbs (0.75 mg GAE·g^{-1}). 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 sulfur-containing 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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References

Allina SM, Pri-Hadash A, Theilmann DA, Ellis BE, Douglas CJ (1998) 4-Coumarate:coenzyme a ligase in hybrid poplar. properties of native enzymes, cDNA cloning, and analysis of recombinant enzymes. Plant Physiol 116:743-754

Báidez AG, Gómez P, Río JAD, Ortuño A (2007) Dysfunctionality of the xylem in *Olea europaea* L. plants associated with the infection process by *Verticillium dahliae* Kleb. role of phenolic compounds in plant defense mechanism. J Agric Food Chem 55:3373-3377

Becker-André M, Schulze-Lefert P, Hahlbrock K (1991) Structural comparison, modes of expression, and putative cis-acting elements of the two 4-coumarate: CoA ligase genes in potato. J Biol Chem 266:8551-8559

Bell-Lelong DA, Cusumano JC, Meyer K, Chapple C (1997) Cinnamate-4-hydroxylase expression in Arabidopsis (regulation in response to development and the environment). Plant Physiol 113:729-738

Benavente-Garc O, Castillo J, Lorente J, Ortu A, Del Rio JA (2000) Antioxidant activity of phenolics extracted from *Olea europaea* L. leaves. Food Chem 68:457-462

Bhagyalakshmi N, Thimmaraju R, Venkatachalam L, Murthy KNC, Sreedhar RV (2005) Nutraceutical applications of garlic and the intervention of biotechnology. Crit Rev Food Sci Nutr 45:607 - 621

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

Dixon RA, Maxwell CA, Weiting N, Oommen A, Paiva NL (1994) Genetic manipulation of lignin and phenylpropanoid compounds involved with microorganisms. In: Ellis BE, Kuroki GW, Stafford HH (eds) Genetic Engineering of Plant Secondary Metabolsim. Plenum Press, New York, pp 153-178

Douglas CJ, Hauffe KD, Ites-Morales ME, Ellard M, Paszkowski U, Hahlbrock K, Dangl JL (1991) Exonic sequences are required for elicitor and light activation of a plant defense gene, but promoter sequences are sufficient for tissue specific expression. EMBO J 10:1767-1775

Ehlting J, Büttner D, Wang Q, Douglas CJ, Somssich IE, Kombrink E (1999) Three 4-coumarate:coenzyme A ligases in Arabidopsis thaliana represent two evolutionarily divergent classes in angiosperms. Plant J 19:9-20

Ehlting J, Shin JJK, Douglas CJ (2001) Identification of 4-coumarate:coenzyme A ligase (4CL) substrate recognition domains. Plant J 27:455-465

Ellard-Ivey M, Douglas CJ (1996) Role of jasmonates in the elicitor- and wound-inducible expression of defense genes in parsley and transgenic tobacco. Plant Physiol 112:183-192

Han X, Shen T, Lou H (2007) Dietary polyphenols and their biological significance. Int J Mol Sci 8:950-988

Hu W-J, Harding SA, Lung J, Popko JL, Ralph J, Stokke DD, Tsai C-J, Chiang VL (1999) Repression of lignin biosynthesis promotes cellulose accumulation and growth in transgenic trees. Nat Biotech 17:808-812

Kajita S, Hishiyama S, Tomimura Y, Katayama Y, Omori S (1997) Structural characterization of modified lignin in transgenic tobacco plants in which the activity of 4-coumarate:coenzyme A ligase Is depressed. Plant Physiol 114:871-879

Lee D, Meyer K, Chapple C, Douglas CJ (1997) Antisense suppression of 4-coumarate:coenzyme A ligase activity in Arabidopsis leads to altered lignin subunit composition. Plant Cell 9:1985-1998

Li L, Zhou Y, Cheng X, Sun J, Marita JM, Ralph J, Chiang VL (2003) Combinatorial modification of multiple lignin traits in trees through multigene cotransformation. Proc Nat Acad Sci USA 100:4939-4944

Logemann E, Parniske M, Hahlbrock K (1995) Modes of expression and common structural features of the complete phenylalanine ammonia-lyase gene family in parsley. Proc Nat Acad Sci USA 92:5905-5909

Manach C, Mazur A, Scalbert A (2005) Polyphenols and prevention of cardiovascular diseases. Curr Opin Lipid 16:77-84

Milner JA (2001) A Historical perspective on garlic and cancer. J Nutr 131:1027S-1031

Nasim SA, Dhir B, Samar F, Rashmi K, Mahmooduzzafar, Mujib A (2009) Sulphur treatment alters the therapeutic potency of alliin obtained from garlic leaf extract. Food Chem Toxicol 47:888-892

Owen RW, Giacosa A, Hull WE, Haubner R, Spiegelhalder B, Bartsch H (2000) The antioxidant/anticancer potential of phenolic compounds isolated from olive oil. Eur J Cancer 36:1235-1247

Park SY, Yoo SS, Shim JH, Chin KB (2008) Physicochemical properties, and antioxidant and antimicrobial effects of garlic and onion powder in fresh pork belly and loin during refrigerated Storage. J Food Sci 73:C577-C584

Singleton VL, Orthofer R, Lamuela-Ravent RM, Lester P (1999) Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. In: Methods in Enzymology. Academic Press, pp 152-178

Uhlmann A, Ebel J (1993) Molecular cloning and expression of 4-coumarate:coenzyme A ligase, an enzyme involved in the resistance response of soybean (*Glycine max* L.) against Pathogen Attack. Plant Physiol 102:1147-1156

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