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 pharmacological 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 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 (Bhagyakshmi 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’-ARCGTGNGAYGGNGAVYCCBA-3’) and reverse (5’-ASCCATTWWATTGATTAVDYTCCT-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’-GCTAAGGAGCCTTTTGTGAAAT-3’) and As4CL_5’
(5′-CCTCCAGATCGTTCCATGTTTCTT-3′). Finally, the PCR products were purified and cloned into a T-blunt vector (SolGent, Daejeon, Korea) 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′-AGATGGTGATCGCTACGGGGATA-3′) and As4CL_R (5′-CTCTCAGCCATCTCTATCTTTCA-3′) by using the SYBR Green Realtime PCR Master Mix kit (Toyobo). We also used the primers AsACTIN_F (5′-TGTCTCGTGTAGGAGAGAG-3′) and AsACTIN_R (5′-AGCTCGTGTTTGAAGAGGTGAT-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 mg mL\(^{-1}\)) was added to 1.5 mL of distilled deionized water (DDW), and then mixed with 100 µL of 2 N Folin-Ciocalteu reagent (Sigma, St Louis, Mo). After 5 min, the reaction was neutralized with 1.5 mL of 7.5% Na\(_2\)CO\(_3\), and then incubated at 30 °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\(^{-1}\) dry weight. All measurements were performed in triplicate.
different organs of garlic ranged from 0.75 to 6.01 mg GAE/g. Phenolic compounds were more abundant in the leaves and bulbs (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-Leelong 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**


