# Cloning and Characterization of Phenylalanine Ammonia-Lyase and Cinnamate 4-Hydroxylase and Pyranocoumarin Biosynthesis in *Angelica gigas*

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Phenylalanine ammonia-lyase (PAL) and cinnamate 4-hydroxylase (C4H) are important enzymes in the phenylpropanoid pathway and also in the accumulation of decursin (**1**) and decursinol angelate (**2**), which are major secondary metabolites in *Angelica gigas*. Using PCR with degenerate primers targeted to conserved regions of available orthologous *PAL* and *C4H* sequences, cDNAs encoding PAL and C4H from *A. gigas* were isolated. Both genes were used to show the comparative developmental and inducible accumulation of mRNAs in different organs and in suspension cells of *A. gigas*. PAL and C4H were induced most strongly in response to 300 $\mu$M methyl jasmonate treatment at 6 and 12 h, respectively, and were highly expressed in the fine roots of *A. gigas*. Similarly, the production of **1** and **2** was most prolific in the fine roots of the plant.

*Angelica gigas* Nakai (Apiaceae), generally known as Korean angelica, is one of the most popular medicinal plants in Korea. This plant is distinctive in that it bears dark purple flowers, unlike Chinese and Japanese angelica flowers, which are white.[1] The roots of *A. gigas* have been used widely in traditional oriental herbal medicine to treat abdominal pain, injuries, migraine, arthritis, and female afflictions such as anemia and dysmenorrhea.[2,3] It has been reported that *A. gigas* biosynthesizes a variety of secondary metabolites, including coumarins.[4] The roots of *A. gigas* contain decursin (**1**), decursinol, imperatorin, nodakenin, nodakenetin, and umbelliferone, whereas the fruits accumulate decursinol, decursidin, and imperatorin.[2] Decursin (**1**) and decursinol angelate (**2**) (Figure 1), pyranocoumarins isolated from *A. gigas* roots, are the major secondary metabolites of this species, and these two compounds are structural isomers of one another. It has been reported that **1** and **2** exhibit anti-androgen receptor signaling, cytotoxic, and neuroprotective activities.[5−7] Three different angelica species, namely, Korean angelica (*A. gigas*), Chinese angelica (*A. sinensis* Oliv.), and Japanese angelica [*A. acutiloba* (Sieb. et Zucc.) Kitag.], are considered to contain different amounts of the various above-mentioned compounds.[8,9] However, Korean angelica contains greater amounts of **1** and **2** than the other two species.

The phenylpropanoid pathway is an important secondary metabolic pathway in plants and produces a large number of various physiologically important metabolites.[10] Phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) is the first key enzyme in the phenylpropanoid pathway and catalyzes the conversion of L-phenylalanine to *trans*-cinnamic acid.[11] PAL is an extensively studied enzyme in plants because of its importance in the biosynthesis of various secondary metabolites. In recent years, researchers have cloned PAL genes from different plant species and determined their sequences.[11−15] PAL has been proposed as a key enzyme that carries out many essential functions involved in mechanical support and the production of pigments such as anthocyanins and flavonoid nodulation factors.[16−18] PAL also induces phenylpropanoid biosynthesis in response to biotic and abiotic stress, inclusive of pathogen attack, UV irradiation, mechanical wounding, and light.[10] Cinnamate 4-hydroxylase (C4H, EC 1.14.13.11) is a second key enzyme in the phenylpropanoid pathway and catalyzes the hydroxylation of *trans*-cinnamic acid to *p*-coumaric acid.[19] C4H is a member of the CYP73 family of cytochrome P450 monooxygenases, which in plants are involved in the biosynthesis of diverse metabolites.[20] C4H controls the carbon flux for many phytoalexins that are

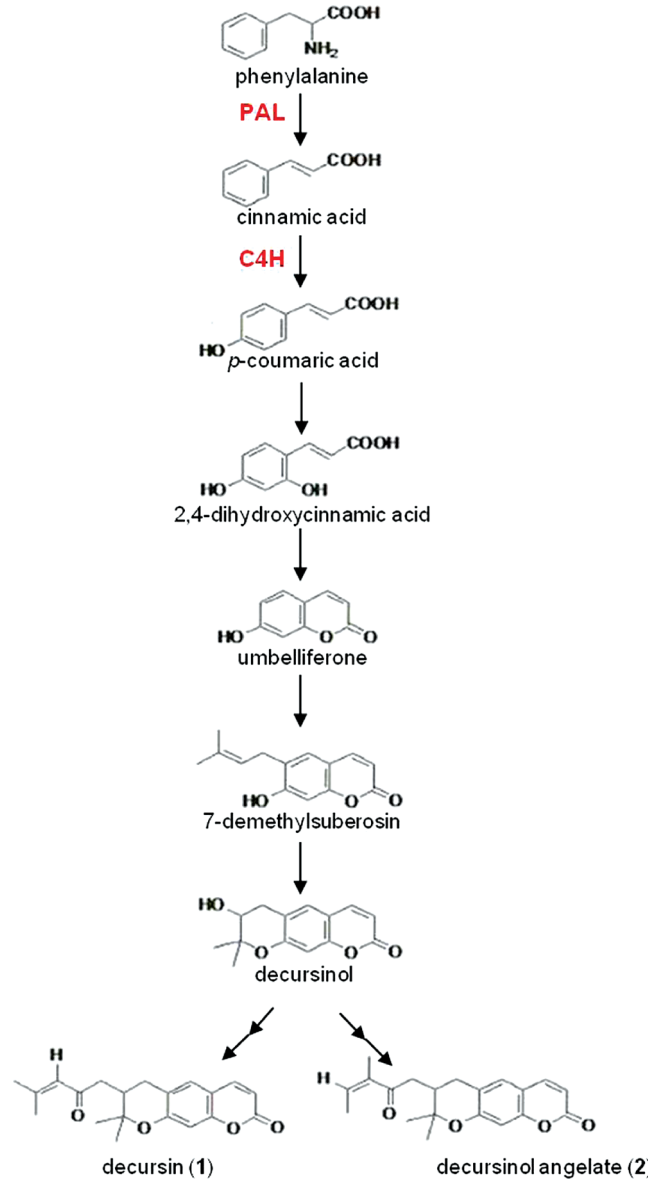


**Figure 1.** Proposed decursin (**1**) and decursinol angelate (**2**) biosynthetic pathway. PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase.

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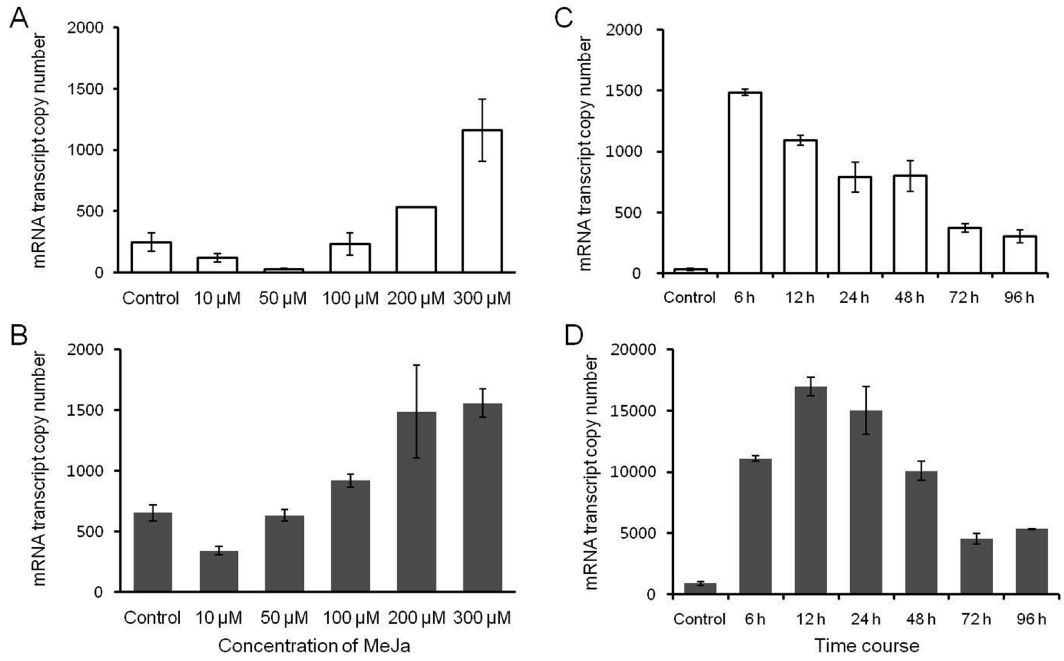


**Figure 2.** Accumulation of mRNA transcripts after treatment with methyl jasmonate in suspension cell cultures of *A. gigas*. Inducible expression of (A) *AgPAL* and (B) *AgC4H* when treated with different concentrations of methyl jasmonate. Inducible expression of (C) *AgPAL* and (D) *AgC4H* over time in suspension cell cultures of *A. gigas* treated with 300 $\mu$M methyl jasmonate.

synthesized when plants are challenged by pathogens and, in a similar manner to PAL, when induced by certain factors.[21]

In the present study, the cDNAs of PAL and C4H from *A. gigas* (*AgPAL* and *AgC4H*; GenBank accession numbers HM114215 and HM114216, respectively) were isolated for the first time. In addition, gene transcript levels were analyzed by quantitative real-time PCR after elicitor treatment of *A. gigas* cell cultures using different concentrations of methyl jasmonate (MeJa) and for different periods of time. The transcript levels of these genes in the different organs of *A. gigas* were also examined. Furthermore, the production of **1** and **2** was analyzed in different organs of *A. gigas*.

## Results and Discussion

**Isolation and Sequence Analysis of PAL and C4H from *A. gigas*.** cDNAs from *A. gigas* encoding PAL and C4H were obtained from RACE PCRs using primers designed from conserved sequences of known PAL and C4H genes. The AgPAL cDNA consisted of a 2100-bp open reading frame (ORF) encoding 699 amino acids with a theoretical molecular mass of 76.02 kDa. From an alignment of the PAL amino acid sequences, AgPAL was found to share 92% identity with PcPAL [*Petroselinum crispum* (Mill.) Fuss subsp. *tuberosum* (Bernh. ex Rahb.) Soó; P45728], 87% identity with DcPAL (*Daucus carota* L.; BAC56977), 80% identity with CsPAL (*Camellia sinensis* (L.) Kuntze; P45726), 79% identity with CrPAL (*Catharanthus roseus* (L.) G. Don; BAA95629), and 78% identity with NtPAL (*Nicotiana tabacum* L.; P45733) (Figure S1a, Supporting Information).

AgC4H was obtained from *A. gigas* using the same method as used for AgPAL. AgC4H cDNA consisted of a 1518-bp ORF encoding 505 amino acids with a theoretical molecular mass of 57.87 kDa. The amino acid sequence of AgC4H showed 93% identity with AmC4H (*Ammi majus* L.; AAO62904), 78% identity with PtC4H (*Populus trichocarpa* Torr. & A. Gray; ACC63873), 75% identity with NtC4H (*Nicotiana tabacum* L.; ABC69412), and 74% identity with RoC4H (*Rubus occidentalis* L.; ACM17896) (Figure S1b, Supporting Information).

**Inducible Expression of AgPAL and AgC4H in Suspension Cells of *A. gigas*.** The transcripts of AgPAL and AgC4H in *A. gigas* were induced in response to MeJa as an elicitor treatment. The expression of AgPAL and AgC4H was examined after 24 h following treatment with 0, 10, 50, 100, 200, and 300 $\mu$M MeJa. The expression of both AgPAL and AgC4H genes was obviously increased after treatment with MeJa, with the highest expression levels being observed in the 300 $\mu$M MeJa treatment. The levels of mRNA transcript for both genes showed a similar pattern (Figure 2A and B).

The accumulation of *AgPAL* and *AgC4H* transcripts was monitored over 0, 6, 12, 24, 48, 72, and 96 h periods after treatment with 300 $\mu$M MeJa. *AgPAL* and *AgC4H* transcripts were induced maximally at 6 and 12 h, respectively, after which the transcript levels gradually decreased (Figure 2C and D). MeJa plays an essential role in the elicitation procedure, causing either immediate or circuitous activation of the genes involved in secondary metabolism. Treatment of certain plants with MeJa activates the induction of genes and secondary metabolism. For example, *Panax ginseng* C. A. Mey is one of the most well-known medicinal plants in Korea, and application of MeJa stimulates many secondary metabolites in this plant[22] and also enhances the ginsenoside content in cell culture.[23] In addition, genes related to secondary metabolites obviously accumulate following treatment with MeJa.[24]

**Developmental Expression of *AgPAL* and *AgC4H* Genes in *A. gigas*.** Quantitative real-time PCR was conducted to analyze the transcript levels of AgPAL and AgC4H in different organs of *A. gigas*: bud, immature flower (IF), mature flower (MF), leaf, stem, primary root (R1), secondary root (R2), and fine root (R3) (Figure 3A). The highest levels of both PAL and C4H transcripts were observed in the roots (particularly in the fine roots), although lower levels of transcript were detected in the stems. The levels of AgPAL and AgC4H mRNA transcripts showed a similar pattern (Figure 3B and C).

**HPLC Analysis of Decursin (1) and Decursinol Angelate (2) in *A. gigas* Organs.** The decursin (**1**) and decursinol angelate (**2**) content of *A. gigas* varied among the different organs studied, as judged from HPLC analyses (Figure S2, Supporting Information). The respective retention times of **1** and **2** with the standard were almost the same at 20 and 21 min, respectively. The aerial parts of *A. gigas* bud, immature flower, mature flower, and leaf contained greater amounts of **2** than **1**, whereas in the stem the concentration

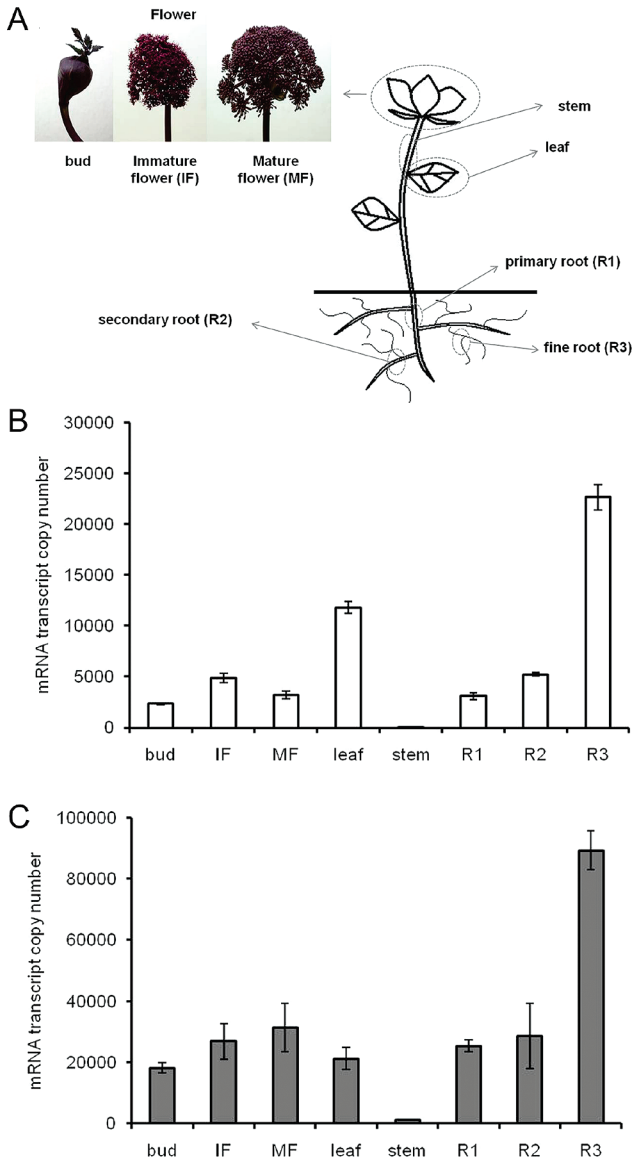


**Figure 3.** Schematic of organs and developmental gene expression in different parts of *A. gigas*. (A) Schematic of the organs of *A. gigas* examined in this study. Quantitative RT-PCR analysis of (B) *AgPAL* and (C) *AgC4H* gene expression in different organs (bud; IF, immature flower; MF, mature flower; leaf; stem; R1, primary root; R2, secondary root; R3, fine root) of *A. gigas.*

levels of these two compounds were very similar. Conversely, in the underground parts (primary root, secondary root, and fine root) the amounts of **1** were higher than those of **2**. Moreover, the fine roots had the largest amounts of **1** and **2** among all the organs of *A. gigas* examined (Figure 4). Suspension cells did not contain **1** and **2** (data not shown). The *AgPAL* and *AgC4H* genes isolated in the present study were highly expressed in the fine roots (R3) of *A. gigas* and to a greater extent than in the other organs examined; moreover, the content of **1** and **2** was also highest in the fine roots. These results indicate that the expression of *AgPAL* and *AgC4H* genes is associated with the production of secondary metabolites in *A. gigas*. Therefore, it can be expected that the overexpression of the *AgPAL* and *AgC4H* genes will have the effect of increasing the secondary metabolite levels of this plant. This suggests the potential of producing further secondary metabolites using hairy root cultures of *A. gigas*. Transformed hairy root cultures have attracted considerable attention owing to their genetic and biochemical stability, rapid growth rate, and ability to synthesize secondary products at levels comparable to wild-type roots.[25,26] The isolation of genes encoding biosynthetic enzymes coupled with hairy root culture will provide a powerful model system for investigating molecular regulation and for evaluating the potential of metabolic engineering.

The phytochemicals examined, here decursin (**1**) and decursinol angelate (**2**), are only some of the many compounds produced by *A. gigas*; its rich supply of secondary metabolites and its importance as a medicinal plant make it an excellent model system for continued research of useful bioactive compounds. To determine that AgPAL and AgC4H are key enzymes in the *A. gigas* biosynthetic pathway, our group is currently focusing its efforts on producing transgenic hairy roots via the introduction of biosynthetic genes. Cumulatively, it is hoped that this and related work will lead to the development of improved technology for producing useful medicinal compounds.

## Experimental Section

**Plant Material.** *Angelica gigas* Nakai was grown in a glasshouse at the experimental farm of Chungnam National University (Daejeon, Republic of Korea). This plant was obtained from the Department of Herbal Crop Research, Institute of Horticultural and Herbal Science, Suwon, Republic of Korea. A voucher specimen is deposited at the Department of Crop Science, Chungnam National University, Daejeon, Republic of Korea. Plants were collected and stored at −80 °C. Prior to experiments, each organ of *A. gigas* (e.g., flowers, stems, leaves, roots) was ground with a mortar and pestle under liquid nitrogen. For preparation of suspension cell cultures, the seeds were placed on agar-solidified MS medium and germinated in a growth chamber at 25 °C, and then the roots from seedlings (30 days after germination) were used.

**Maintenance of Plant Cell Suspension Cultures.** *A. gigas* cell suspension cultures were grown in B5 medium consisting of Gamborg B5 salts and vitamins plus 30 g/L sucrose and 2 mg/L 2,4-D. Cells were subcultured every 7 days and maintained in the dark at 25 °C on a gyratory shaker (120 rpm) in a growth chamber under a 16 h photoperiod.

**Preparation and Use of Elicitors.** Methyl jasmonate was dissolved initially in 100% ethanol at an optimal concentration. Elicitor solutions were added immediately to the cell cultures at concentrations of 0, 10, 50, 100, 200, and 300 $\mu$M for 24 h for quantitative real-time PCR analysis. To examine time effects, suspension cells were treated with 300 $\mu$M methyl jasmonate under the same conditions as described above for 0, 1, 3, 6, 12, 24, 48, 72, and 96 h. Plant suspension cells treated with elicitor were collected and stored frozen in sealed clear polyethylene plastic bags at −80 °C until they were used. Each treatment consisted of three flasks, and the experiment was repeated three times.

**Total RNA Isolation and cDNA Synthesis.** Total RNA was extracted from different tissues of *A. gigas*. Tissue aliquots (100 mg) were ground with a mortar and pestle under liquid nitrogen followed by the addition of 1 mL of TRIZOL (Invitrogen, Carlsbad, CA). After incubation for 15 min at room temperature, 200 $\mu$L of chloroform per 1 mL of TRIZOL was added, and the solution was vortexed for 15 s and then incubated for 10 min at room temperature. After centrifugation at 12 000 rpm for 15 min at 4 °C, the supernatants were transferred to fresh tubes followed by the addition of 500 $\mu$L of 2-propanol, gentle mixing, and incubation for 10 min at room temperature. After centrifugation at 12 000 rpm for 10 min at 4 °C, total RNA was precipitated, forming a gel-like pellet at the bottom of the tube. RNA pellets were washed two times with 1 mL of ice-cold 75% ethanol. The pellets were dissolved in DEPC water, and then the quality and concentration of the RNA samples were determined by agarose gel electrophoresis and spectrophotometeric analysis. Total RNA (1 $\mu$g) was reverse-transcribed according to the manufacturer's protocol (ReverTra Ace-α; Toyobo, Japan) using an oligo$(dT)_{20}$ primer. The resulting cDNA mixtures were used as templates for RT-PCR and RACE (rapid amplification of cDNA ends) PCR.

**Isolation of cDNA Encoding AgPAL and AgC4H.** To isolate cDNA encoding PAL and C4H, a GeneRacer kit (Invitrogen) was used to synthesize single-strand cDNA using the manufacturer's protocol. All primers for RACE were designed on the basis of the core sequences. 5′-RACE PCR was performed with the GeneRacer 5′ primer and the reverse primer, whereas 3′-RACE PCR was performed using the GeneRacer 3′ primer and the forward primer of each gene. The primers of each full-length gene are listed in Table S1 (Supporting Information).

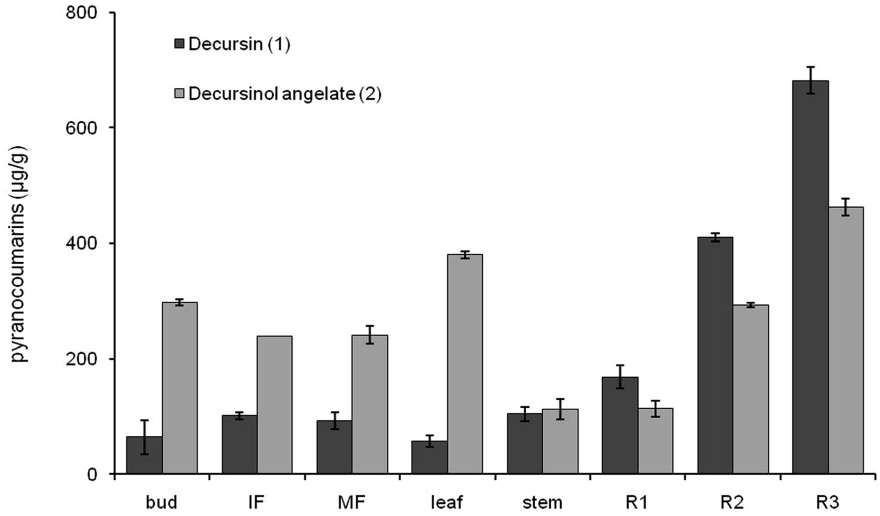


**Figure 4.** Decursin (**1**) and decursinol angelate (**2**) production in different organs (bud; IF, immature flower; MF, mature flower; leaf; stem; R1, primary root; R2, secondary root; R3, fine root) of *A. gigas.*

Accurate annealing temperatures for PCR were calculated using the TM Calculator program (http://www.genotech.co.kr). The PCR products were purified using a Zymoclean Gel and DNA Recovery kit (Zymo Research), cloned into a pGEM-T Easy vector (Promega, Madison, WI), and finally sequenced.

**Quantitative Real-Time PCR.** Quantitative real-time PCR was performed for transcription level analysis of PAL and C4H from *A. gigas* using a Mini Opticon real-time PCR system (BioRad, Hercules, CA). Each pair of specific primers designed from PAL and C4H full-length cDNA sequences were highly specific for each gene, as confirmed by an online program (http://frodo.wi.mit.edu/primer3). Real-time PCR was carried out in 20 $\mu$L reaction volumes containing 0.4 $\mu$M of each primer and 1× SYBR Green real-time PCR master mix (Toyobo). Amplification was conducted as recommended by the manufacturer using modified cycling parameters. Triplicate quantitative real-time PCR experiments were performed for each sample.

**Sequence Alignments.** For sequence alignment, the BioEdit Sequence Alignment Editor V 5.0.9 (Department of Microbiology, North Carolina State University) was used.

**HPLC Analysis of Decursin (1) and Decursinol Angelate (2).** HPLC analysis was carried out using a Futecs model NS-4000 HPLC system (Daejeon, Korea) equipped with autosampler and system connected to a UV detector. Peak area integration was performed using chromatographic data (Multicro 2000). Separation was conducted with a reversed-phase OptimaPak $C_{18}$ (5 $\mu$m, 250 mm × 4.6 mm) column at an oven temperature of 35 °C. The composition of the mobile phase used was acetonitrile 40%, water 50%, and tetrahydrofuran 10%. The flow rate was 0.8 mL/min, the detection wavelength, 280 nm, and the injection volume, 10 $\mu$L.

Decursin (**1**) and decursinol angelate (**2**) standards (95% purity, respectively) were provided by the Plant Natural Product Laboratory of the Korea Research Institute of Chemical Technology (Deajon, Korea). The distilled water, acetonitrile, and tetrahydrofuran used for the mobile phase were purchased from Burdick and Jackson (Morrison, NJ). Samples were dried at −80 °C for 48 h using a freeze-dryer. Dried samples were ground into a fine powder using a mortar and pestle. Samples (0.5 g) were extracted with 30 mL of 70% (v/v) ethanol at 50 °C in a water bath for 1 h (×3). After centrifugation, the supernatant (25 mL) was concentrated under vacuum and extracted three times with methylene chloride. The methylene chloride fraction was dried under vacuum and dissolved in 1 mL of acetonitrile. Samples were filtered through 0.45 $\mu$m poly filters.

**Supporting Information Available:** Table of primers used and figures of the alignment of amino acid sequences for phenylalanine ammonia-lyase (PAL) and cinnamate 4-hydroxylase (C4H), and a HPLC chromatogram. This information is available free of charge via the Internet at http://pubs.acs.org.

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