Molecular Cloning and Characterization of *Cinnamate-4-Hydroxylase* Gene from *Rubus coreanus*

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Abstract: Cinnamate-4-hydroxylase (C4H) is a key enzyme in the phenylpropanoid pathway, which synthesizes a variety of secondary metabolites to participate in differentiation and protection of plant tissues against environmental stresses. We isolated a full-length cDNA of the *C4H* gene from a Korean native bramble (*Rubus coreanus* Mique), using a reverse transcriptase-PCR and a rapid amplification of the cDNA ends (RACE)-PCR. The full-length cDNA of the *RcoC4H* gene contained a 1,515 bp open reading frame (ORF) encoding a 504 amino acid protein with a calculated molecular weight of about 57.9 kDa and an isoelectric point (p*I*) value of 9.1. The genomic DNA analysis revealed that the *RcoC4H* gene had three exons and two introns. The comparison of the deduced amino acid sequence of RcoC4H with other C4Hs was highly conserved among widely divergent plant species. Also, the P450-featured motifs such as the heme-binding domain, the T-containing binding pocket motif (AAIETT), the ERR triad and the tetrapeptide (PPGP) hinge motif necessary for an optimal orientation of the enzyme were highly conserved. Southern blot analysis indicated that *RcoC4H* exists as a single copy in *R. coreanus*. Reverse transcriptase PCR analysis showed that the gene is expressed at similar levels in the stem, leaf and flower.

Keywords: Cinnamate-4-hydroxylase, phenylpropanoid pathway, RACE, *Rubus coreanus*, southern blot.

INTRODUCTION

Rubus coreanus Miquel (Rosaceae) is a Korean raspberry distributed in southern parts of the Korea, China and Japan. The unripe fruits of Korean raspberry have been used as a traditional medicine for the treatment of impotence, spermatorrhea, enuresis, asthma, allergic diseases, and it also has been used as a stomachic and tonic in Korea [1]. Recently, it was reported that berry extracts inhibit the growth and induce apoptosis of different human cancer cell lines *in vitro* [2]. The biological activities of berries are partially determined by their content of a diverse range of phytochemicals such as flavonoids (anthocyanins, flavonols, and flavanols), tannins (proanthocyanidins, ellagitannins, and gallotannins), stilbenoids (e.g., resveratrol), phenolic acids (hydroxybenzoic and hydroxycinnamic acid derivatives), and lignins [3, 4]. The quality of ripe raspberry fruits is critically dependent on the accumulation of specific anthocyanin pigments and characteristic flavor components which are biosynthetically produced in the phenylpropanoid pathway [4-6].

Cinnamate 4-hydroxylase (C4H) is a member of the cytochrome P450 monooxygenase superfamily and it catalyzes the first oxygenation step during a phenylpropanoid metabolism, the hydroxylation of *trans*-cinnamate to *p*-coumarate (4-hydroxy *trans*-cinnamate), and the phenylpropanoid biosynthesis branch pathways lead to the synthesis of a variety of compounds including lignin monomers, flavonoids, hydroxycinnamic esters and coumarins. These compounds are necessary for UV-protection, differentiation of tissues and defense giving reason for the induction of C4H upon irradiation, wounding, elicitors, and pathogen infection of plants [7]. In addition, C4H is regulated in a pattern of temporal and spatial gene expression correlated with its role in lignifications [8, 9].

Many C4Hs have been isolated and characterized in herbal plants. However, there are scanty reports about the cloning of *C4H* genes from woody plant species. In woody plants, C4H is particularly important for the biosynthesis of lignin, the second most abundant biopolymer after cellulose, and C4H is likely to play a pivotal role in the ability of a phenylpropanoid metabolism to channel carbon from a primary metabolism into a biosynthesis of lignin and other polymers in trees. The *C4H* gene from raspberry which has been used as a traditional Korean medicinal woody-plant was firstly isolated in this study. We report on the cloning and characterization of a full-length cDNA sequence of the *C4H* gene from *R. coreanus*. This report will be helpful to further research the role of the *RcoC4H* gene during a phenylpropanoid metabolism in *R. coreanus*.

MATERIALS AND METHODS

Plant Growth Conditions and Materials

Korean native bramble (*Rubus coreanus* Miq.) was grown in experimental plots at the Korean Black Raspberry Research Center, Gochang, Republic of Korea, under ambient conditions. The leaf, stem and flower were collected

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separately, immediately frozen in liquid nitrogen and stored at -70ºC until used.

Isolation of the Full-Length cDNA and Genomic DNA of *RcoC4H*

Total RNA of each tissue sample was isolated using a cetyltrimethylammonium bromide (CTAB) method [10]. To isolate the full-length cDNA of *RcoC4H*, 5′- and 3′-RACE were performed using a gene-specific primer according to the SMART™ RACE cDNA Amplification Kit (Clontech Laboratories, CA, USA) by following the manufacture's instruction. Two gene-specific primers, RcoC4H-GSP1 (5′-CAGGACATGGTGTTCACCGTCTACGG-3′) and Rco C4H-GSP2 (5′-GGTTCACAAGCTCGGCAATTCCCCA -3′), were designed and synthesized based on the conserved sequences of other *C4H* genes. RACE-PCR conditions were 5 cycles of 94ºC for 30 sec, 72ºC for 2 min, then 5 cycles of 94ºC for 30 sec, 70ºC for 30 sec, 72ºC for 2 min, and 30 cycles of 94ºC for 30 sec, 68ºC for 30 sec, 72ºC for 2 min. The amplified RACE-PCR products were purified, ligated into the pGEM-T Easy vector (Promega, Madison, USA) and cloned into *E. coli* strain XL1-blue MRF′ followed by a sequencing. After comparing and aligning the sequences of the 5′ and 3′ RACE-PCR products, the cDNA of *RcoC4H* was obtained through a reverse transcriptase (RT)-PCR, with RcoC4H-F (5′-catatgGATCTCCTCCTCATGGAGAAG AC-3′, the restriction endonuclease site was represented by lower case letters and the start codon was boxed) and RcoC4H-R (5′-ctcgagTTATGTCCTTGGCTTCATGACT ATG-3', the restriction endonuclease site was represented by lower case letters and the stop codon was boxed) as primers, and the cDNA library prepared by a *Maxime* RT premix kit (iNtRON BIOTECHNOLOGY, Seongnam, Korea) as a template. The PCR was carried out using *Maxime* PCR PreMix (*i-MAX* II) (iNtRON BIOTECHNOLOGY, Seongnam, Korea) under the following condition: 94ºC for 30 sec, 63ºC for 30 sec, and 72ºC for 2 min with 30 cycles.

To generate the genomic DNA of *RcoC4H*, we isolated the genomic DNA from the leaves by a modified CTAB method [11], and then a PCR was performed with the primers of RcoC4H-F and RcoC4H-R covering the coding region under the following condition: 94ºC for 30 sec, 63ºC for 30 sec, and 72ºC for 3 min with 30 cycles. The PCR products were purified, ligated into a pGEM-T Easy vector (Promega, Madison, USA) and cloned into *E. coli* strain XL1-blue MRF′ followed by a sequencing.

Bioinformatic Analysis

The obtained sequences were analyzed using bioinformatics tools from websites (www.ncbi.nlm.nih.gov, www.expasy.org). Multiple sequence alignment of RcoC4H and other plant C4Hs were carried out using the CLUSTAL W program [12] from web sites (www.ebi.ac.uk/Tools/clustalw/index.html, http://www.genome.jp/) and a phylogenetic analysis was constructed by the neighbour-joining method [13, 14] using the CLUSTAL W program.

Southern Blot Analysis

Genomic DNA was isolated from the leaves by a modified CTAB method [11]. Ten-μg aliquots of the total genomic DNA were digested overnight at 37ºC with *Hin*d III, *Xba* I, and *Xho* I (Takara, Shiga, Japan) respectively, and fractioned by 0.8% agarose gel electrophoresis and then transferred onto a positively charged nylon membrane (Zeta-Probe blotting membrane; Bio-Rad, CA, USA) by a capillary transfer [15]. PCR Digoxigenin (DIG) probe synthesis kit (Roche; Mannheim, Germany) was used to label the probe, cDNA fragment including the *RcoC4H* ORF region, with DIG-11-dUTP according to the manufacturer's instructions. Hybridization was carried out at 50°C for overnight in the DIG Easy Hyb (Roche; Mannheim, Germany). After a stringent washing with 2X sodium chloride-sodium citrate (1X SSC = 150 mM NaCl and 15 mM sodium citrate, pH 7.0), 0.1% sodium dodecyl sulfate (SDS) (w/v) twice at room temperature for 5 min and then with 0.1X SSC, 0.1% SDS (w/v) twice at 68°C for 15 min under a constant agitation, the signals were visualized with the DIG Nucleic Acid Detection Kit (Roche; Mannheim, Germany). Photographs of the hybridization signals were taken with the use of a digital camera (Canon, Japan).

Reverse Transcription (RT)-PCR

To investigate the transcription levels of *RcoC4H* in different tissues, the total RNA of each tissue sample was isolated using a CTAB method [10]. One-μg of total RNA was reverse-transcribed in an RT system, the *Maxime* RT premix kit (iNtRON BIOTECHNOLOGY, Seongnam, Korea) for 60 min at 45°C. The PCR reaction was carried out using the *Maxime* PCR PreMix (*i-MAX* II) (iNtRON BIOTECHNOLOGY, Seongnam, Korea) with specific primers of RT-C4Hf (5′-GAGCTTCGAGTACAACTACTACG-3′) and RT-C4Hr (5′-GATCTCTCCTTTCTGCTGAG-3′) under the following conditions: 94ºC for 2 min, 30 cycles of 94ºC for 20 sec, 50ºC for 20 sec, 72ºC for 30 sec, and 72ºC for 5 min. For equal amounts of the total RNA and the efficiency of the cDNA synthesis from different tissues, the intensities of the bands were normalized with an average intensity of the *Rubus* sp. *Histone h3* gene (GenBank accession no. AF304365) products [6]. RT-PCR assays were repeated in triplicate from independent mRNA extractions.

RESULTS AND DISCUSSION

ISOLATION OF THE *C4H* GENE FROM *R. COREANUS*

The full-length cDNA sequence of the *RcoC4H* gene (GenBank accession no. EU123531) was isolated using the RACE-PCR method. It was 1,741 bp, consisting of a 1,515-bp ORF (including stop codon, TAA) encoding a 504 amino acid protein, with 50 bp of the 5′ untranslated region (UTR) and 176 bp of the 3′ UTR including a poly (A) tail. A start codon (ATG) which conforms to the predicted translation start site for eukaryotic genes (A/GXXATGG) [16] is present (Fig. **1A**). The deduced RcoC4H protein encodes a polypeptide of 504 amino acid residues, with a calculated molecular weight of 57.9 kDa and an isoelectric point (p*I*) value of 9.1 (using the software of Compute pI/Mw tool at www.expasy.org).

To isolate the genomic *RcoC4H* gene, we carried out a PCR using specific primers of RcoC4H-F and RcoC4H-R, derived from the start and stop codon regions of the cDNA. A 3,282 bp of genomic *RcoC4H* containing a start and stop codon was isolated, which had a 100% identity in the coding

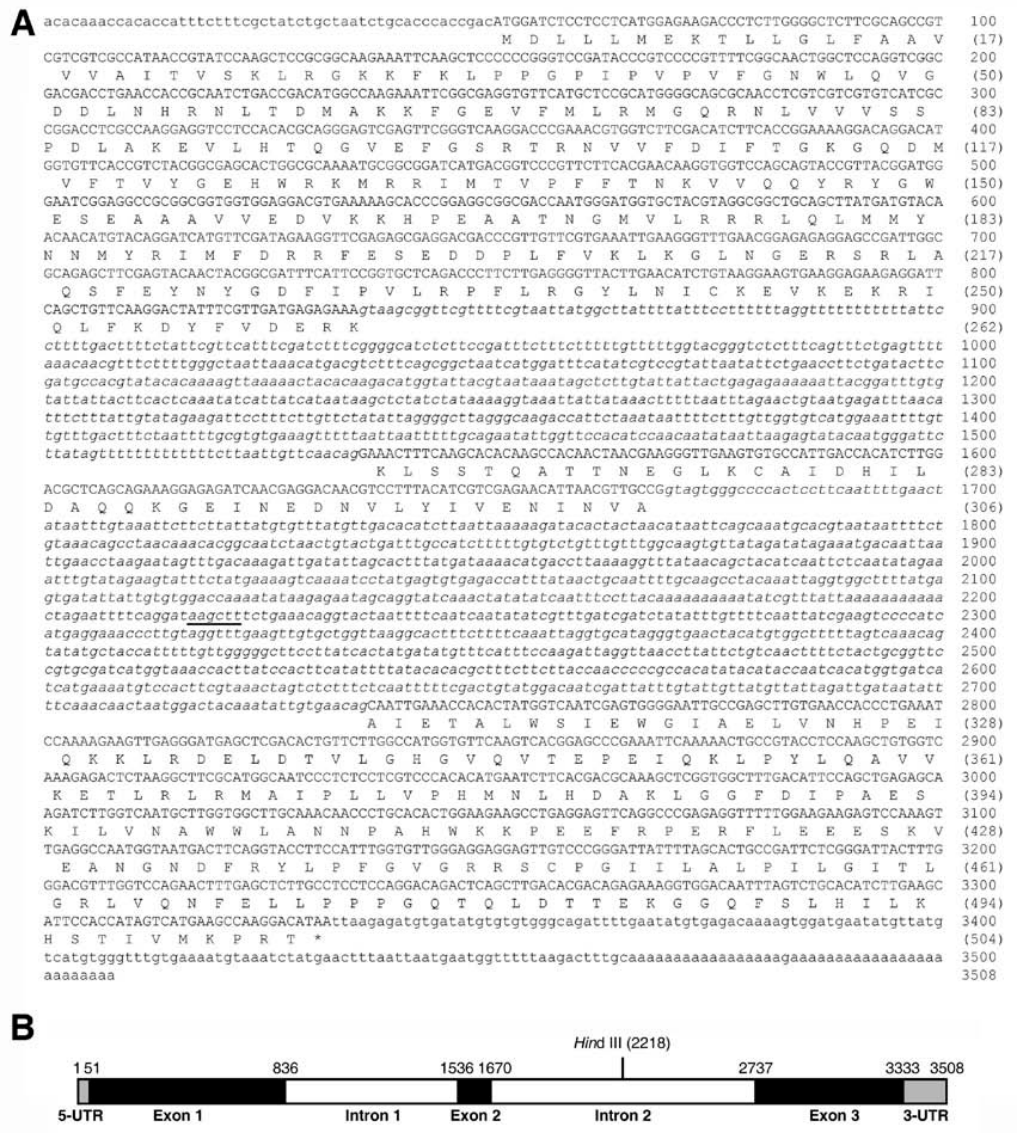

Fig. (1). Sequence and structure of the *RcoC4H* gene: A, The genomic DNA sequence and deduced amino acid sequence of *RcoC4H*. The stop codon (TAA) is represented as an asterisk in the sequence. Lower case letters: UTR, capital letters: translated region, italic lower case letters: intron. *Hin*d III restriction endonuclease site is indicated by an underline. The nucleotides (upper line) and amino acid residues (lower line, in parenthesis) are numbered on the right. B, Schematic representation of the genomic DNA structure of *RcoC4H*. Positions of the exons (black boxes) and introns (white boxes) were determined by comparing the genomic DNA sequence with the cDNAs of *RcoC4H.* Three exons were separated by the white-boxed introns. The gray box represents the UTR.

region to the full-length cDNA sequence. Genomic *RcoC4H* gene contains three exons and two introns (Fig. **1**). It was also found that the putative splicing site obeyed the GU/AG rule (Fig. **1A**).

The translated protein of the isolated cDNA, RcoC4H, shares high sequence homology (about 79%-94%) with other plant C4Hs. Especially, the deduced amino acid sequence of the RcoC4H protein shows 93.7% identity to *Malus* x *domestica* C4H (MdC4H). RcoC4H has generally the P450-featured motifs (Fig. **2**), such as the heme-iron binding domain P_{439}FGVGRRSCPG$_{449}$, the T-containing binding pocket motif A_{306}AIETT$_{311}$, the E_{363}-R_{366}-R_{420} triad, and a conserved P_{34}PGP$_{37}$ tetrapeptide necessary for optimal orientation of the enzyme [7, 17]. The central part of the heme-binding loop contains a proline residue (Pro$_{448}$), essential for heme-protein interactions, typically found in the plant species of C4Hs [18]. And also, the substrate recognition sites (SRS), important for regulating substrate specificities [19, 20] were highly conserved in the plant species of C4Hs. The SRS5 and SRS 6 regions and the C-terminal end of the SRS4 region are important in contacting the aromatic rings of the substrate, and the SRS1 and SRS2 regions and the N-terminal end of the SRS4 are important in contacting the aliphatic regions of the substrate [20].

SOUTHERN BLOT ANALYSIS

To determine the copy number of the *C4H* genes in the genome of *R. coreanus*, genomic DNA was isolated and aliquots of genomic DNA (10 μg/sample) were restricted with *Hin*d III, *Xba* I and *Xho* I respectively. Single *Hin*d III recognition site exists within the genomic *RcoC4H* gene, posi-

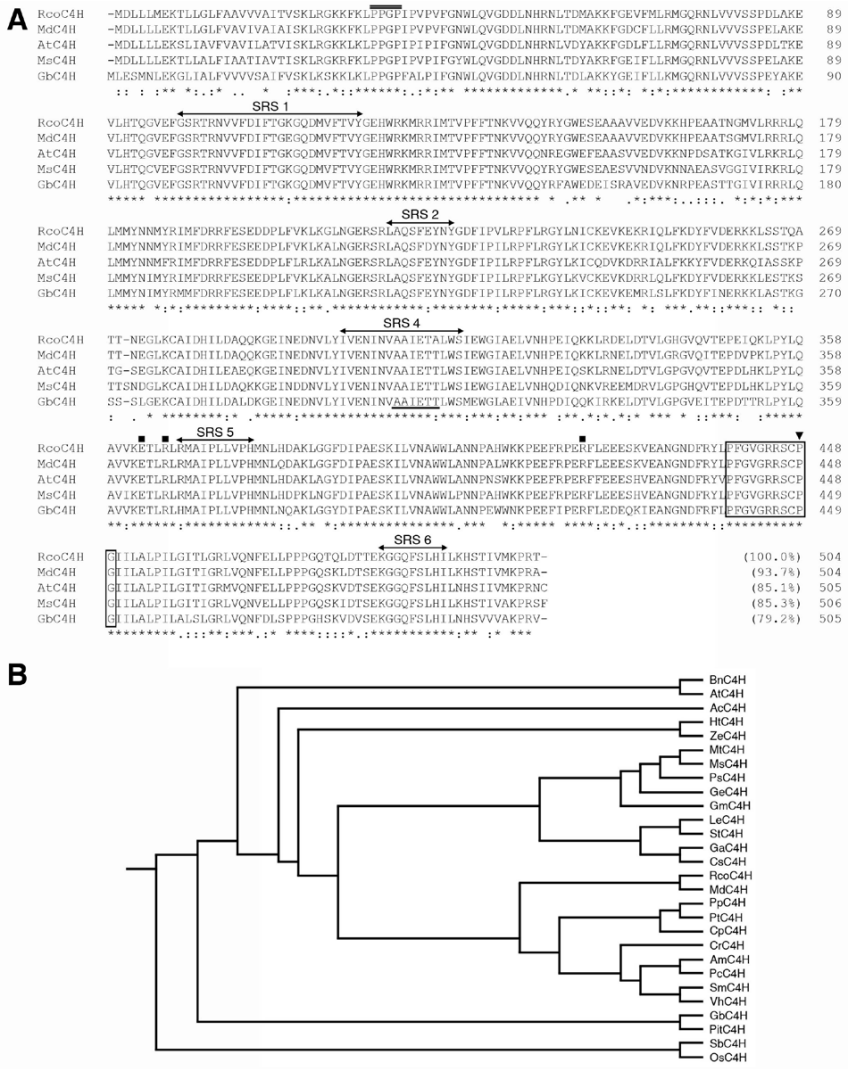

Fig. (2). Comparison of the amino acid sequence of RcoC4H with other plant C4Hs: A, The deduced amino acid sequences of RcoC4H were aligned with those of other plant C4H proteins using the CLUSTAL W method. Amino acid residues are numbered at the right side, and the percentage of the number of identical amino acids shared with RcoC4H is shown in parentheses at the end of the last lines. The Pro-rich domain (PPGP) is indicated by a double-line. The T-containing binding pocket motif (AAIETT) is underlined. ERR tried is indicated by closed square. The heme-binding domain (P-F-G/S-X-G-X-R/H-C-X-G/A/D) is highlighted by a box. SRS domains typical of eukaryotic P450s are indicated by arrow lines labeled. Asterisks indicate the amino acids conserved in all the sequences and the dashes within sequences represent the gaps introduced to optimize alignment. '*' indicates positions which have a single, fully conserved residue. ':' indicates that one of the following 'strong' groups is fully conserved. '.' indicates that one of the following 'weaker' groups is fully conserved. Multiple alignments were analyzed by ClustalW. B, Phylogenetic analysis of RcoC4H and other C4H proteins. A phylogenic tree was constructed by the neighbour-joining method using the CLUSTAL W program. GenBank and Swiss-Prot accession number of the proteins are as follows: MdC4H (*Malus* x *domestica*, AAY87450), SmC4H (*Salvia miltiorrhiza*, ABN48717), GmC4H (*Glycine max*, Q42797), LeC4H (*Lithospermum erythrorhizon*, BAB71716), StC4H (*Solanum tuberosum*, ABC69046), HtC4H (*Helianthus tuberosus*, Q04468), PpC4H (*Populus trichocarpa* x *Populus deltoids*, AAG50231), GaC4H (*Gossypium arboretum*, AAG10197), VhC4H (*Verbena* x *hybrida*, BAE72875), CrC4H (*Catharanthus roseus*, P48522), ZeC4H (*Zinnia elegans*, Q43240), AcC4H (*Allium cepa*, AAS48416), AmC4H (*Ammi majus*, AAO62904), PcC4H (*Petroselinum crispum*, Q43033), GeC4H (*Glycyrrhiza echinata*, Q96423), PtC4H (*Populus tremuloides*, ABF69102), BnC4H (*Brassica napus*, ABF17873), MtC4H (*Medicago truncatula*, ABC59087), CpC4H (*Citrus* x *paradisi*, AAK57011), CsC4H (*Cucumis sativus*, CAK95273), MsC4H (*Medicago sativa*, P37114), AtC4H (*Arabidopsis thaliana*, AAB58355), PsC4H (*Pisum sativum*, Q43067), GbC4H (*Ginkgo biloba*, AAW70021), PitC4H (*Pinus taeda*, AAD23378), SbC4H (*Sorghum bicolor*, AAK54447), OsC4H (*Oryza sativa*, NP_001055190).

tioned at intron 2 (Fig. **1A**), whereas *Xba* I and *Xho* I did not cut into the genomic *RcoC4H* gene. Two bands can be observed in the lanes where DNA was digested with *Hin*d III. However, a single band was detected in the *Xba* I and *Xho* I digested fragment lanes (Fig. **3**). This result suggests that *C4H* exists as a single gene in the *R. coreanus*. Like a *R. coreanus*, some plant genera are assumed to contain only one gene for *C4H*, such as *Pisum* [21], *Arabidopsis* [8, 9, 22], and *Ammi majus* [23], whereas *C4H* genes from alfalfa [24], periwinkle [25], hybrid poplar [26] and Valencia orange [27] encode small gene families.

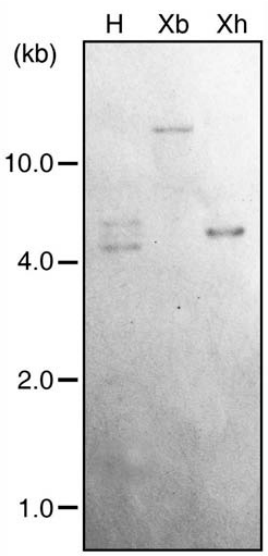

Fig. (3). Genomic southern blot analysis of *RcoC4H*: Genomic DNA of *R. coreanus* was extracted as described in "MATERIALS AND METHODS." The digestion was performed using the restriction enzymes indicated at the top of the lanes: H, *Hin*d III; Xb, *Xba* I; Xh, *Xho* I. The sizes of the molecular weight markers are indicated in kb on the left side.

EXPRESSION PATTERN OF THE *RCOC4H* IN *R. COREANUS*

To investigate the expression pattern of *RcoC4H* in *R. coreanus*, total RNA was isolated from the flowers, stems and leaves, respectively. The RT-PCR analysis was performed using the gene-specific primers of RT-C4Hf and RT-C4Hr. *C4H* mRNA expression was normalized to *Rubus* sp. *histone h3*. The result showed that the *RcoC4H* transcripts could be detected at similar levels in the flower, stem and leaf tissues (Fig. **4**), indicating that *RcoC4H* was ubiquitously expressed in all the tissues examined. The wide-expression patterns of *RcoC4H* resemble *Arabidopsis* [8, 22], hybrid poplar [26] and oilseed rape [17].

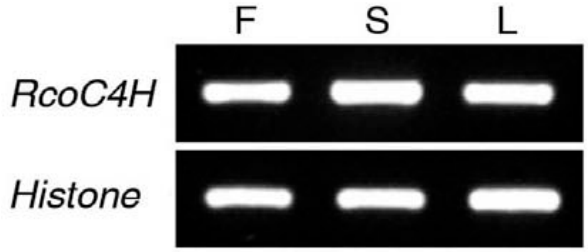

Fig. (4). RT-PCR analysis of the *C4H* expression in *R. coreanus*: Total RNA was extracted from flower (F), stem (S), and leaf (L) tissues. Each RNA sample was used to run the RT-PCR experiments with specific primers as specified in "MATERIALS AND METHODS." The PCR products were separated on an agarose gel. The *histone h3* gene (*Histone*) was used as a quantitative control.

CONCLUSION

In this article, we firstly reported on the isolation and characterization of a full-length cDNA of *C4H* from Korean native raspberry that has been used as a traditional Korean medicinal woody-plant. The full-length cDNA of *RcoC4H* has a 1,515 bp ORF (including stop codon) and the sequence encodes a polypeptide of 504 amino acid residues, with a calculated molecular weight of 57.9 kDa and an isoelectric point (p*I*) value of 9.1. The deduced amino acid sequences of the RcoC4H protein had high identities with other C4Hs. They possess a conserved P450 domain and all the P450-featured motifs. *RcoC4H* exists as a single copy in *R. coreanus* and it is ubiquitously expressed in the flower, stem and leaf tissues. This report will be helpful to further research the role of the *RcoC4H* gene during a phenylpropanoid metabolism in Korean native raspberry.

ACKNOWLEDGEMENTS

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