cDNA cloning and expression pattern of Cinnamate-4-Hydroxylase in the Korean black raspberry

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Cinnamate-4-hydroxylase (C4H) is a key enzyme in the phenylpropanoid pathway, which is responsible for synthesizing a variety of secondary metabolites that participate in development and adaptation. In this study, we isolated a full-length cDNA of the C4H gene from the Korean black raspberry (Rubus sp.) and found that this gene existed as a single gene. By comparing the deduced amino acid sequence of Rubus sp. C4H with other sequences reported previously we determined that this sequence was highly conserved among widely divergent plant species. In addition, quantitative real time PCR studies indicated that the expression pattern during fruit development, where gene expression was first detected in green fruit and was then remarkably reduced in yellow fruit, followed by an increase in red and black fruit. To investigate the two peaks in expression observed during fruit development and ripening, we measured the flavonoid content. The content of the major flavanol of Korean black raspberry fruits was determined to be highest at the beginning of fruit development, followed by a gradually decrease according to the developmental stages. In contrast, the content of anthocyanins during the progress of ripening was dramatically increased. Our results suggest that the C4H gene in Korean black raspberry plays a role during color development at the late stages of fruit ripening, whereas the expression of C4H gene during the early stages may be related to the accumulation of flavanols. [BMB reports 2008; 41(7): 529-536]

INTRODUCTION

The immature ripen fruit of Korean black raspberry has been used in traditional herbal medicine for the management of impotence, spermatorrhea, enuresis, asthma, and allergic disease, whereas the mature fruit has been widely used as processing foods such as wine, juice, jam and cake (1, 2). It has been reported that the functional constituents of the Korean black raspberry fruit are polyphenol, gallic acid, niga-ichigoside F1 and 23-hydroxytormentic acid, which have been shown to display anticarcinogenic, antiniciceptive and anti-inflammatory effects (3, 4).

The phenylpropanoid pathway produces a variety of secondary metabolites such as flavonoids, lignin and hydroxycinnamic acid esters. These secondary metabolites act as signaling molecules or antagonistic components in defense and other physiological activities of plants (5, 6). The quality of ripe raspberry fruits generally comes from the accumulation of specific anthocyanin pigments and characteristic flavour components, which are biosynthetically derived through the phenylpropanoid pathway (7, 8). It has been reported that ripening fruit are associated with the elevated levels of phenylpropanoid derivatives found in other fruits such as Capsicum frutescens (9), Vanilla planifolia (10), Fragaria ananassa (11), and Litchi chinensis (12). The phenylpropanoid derivatives are produced through the general phenylpropanoid pathway and subsequent specialized branches of phenylpropanoid metabolism. Many genes containing the three enzymes; phenylalanine ammonia lyase (PAL), cinnamate-4-hydroxylase (C4H) and 4-coumarate CoA ligase (4CL), which together constitute the general phenylpropanoid pathway, have been isolated and characterized in various plants for the purpose of increasing and/or decreasing the production of certain metabolites (13-16). C4H is the second enzyme in the general phenylpropanoid pathway (the enzyme following PAL) and catalyzes the para-hydroxylation of trans-cinnamic acid. To date, many C4H cDNAs have been isolated from various plants (16-20).

The C4H studies have mainly focused on the biosynthesis of lignin, because it has been reported that the C4H likely plays a key role in the ability of phenylpropanoid metabolism to channel carbon from primary metabolism into the biosynthesis of lignin (15-21). Developmentally regulated C4H expression in parsley is correlated with lignification (17), and the Arabidopsis C4H promoter has been shown to maintain a pattern of temporal and spatial gene expression that is correlated with...
Cinnamate-4-Hydroxylase gene in black raspberry
Myung-Hwa Baek, et al.

Fig. 1. Sequence and structure of the RsC4H gene. (A) Nucleotide and deduced amino acid sequence of RsC4H cDNA. The start codon ATG and the stop codon TAA are represented in bold. The CpG islands are single-lined. The start site for the eukaryotic genes is double-lined, while polyadenylation signal and the pentanucleotide motif in the 3' UTR are dot-lined and boxed, respectively. (B) Schematic representation of the genomic DNA structure of RsC4H. Positions of the exons (black boxes) and introns (white boxes) were determined by comparing the genomic DNA sequence with the cDNAs of RsC4H. Three exons were separated by the white-boxed introns. (C) Multiple-sequence alignment of RsC4H with C4Hs from other plants. Rubus sp (EU123533); Malus x domestica (AAY87450); Parthenocissus henryana (ABA59555); Campotheca acuminata (AAT39513); Lithospermum erythrorhizon (BAB71717); Arabidopsis thaliana (BAA24355). The heme-binding domain was boxed, while the T-containing binding pocket motif and hinge motif were dot-lined and double-lined, respectively and the 5 SRS regions were single-lined. The sequences were aligned using the ClustalW program.
lignification (22). In addition, it has been reported that the C4H expression is rapidly up-regulated by wounding, light, elicitors, and pathogen infection in plants (16). Despite these previous reports, no comprehensive study of the molecular and expression characteristics of C4H during fruit ripening has yet been conducted.

Therefore, in the present study the cloning and characterization of the C4H gene differentially expressed during the fruit ripening of the Korean black raspberry was determined. RT-PCR and quantitative real-time PCR analysis allowed a relationship between the C4H expression pattern and the fruit ripening process to be established. Expression of the RsC4H gene in the early stages of fruit ripening was found to be associated with the synthesis of flavanols in green fruits. In addition, the RsC4H gene also played a role in the biosynthesis of anthocyanin during color development in the late stages of fruit ripening.

RESULTS AND DISCUSSION

Cloning and sequence analysis of RsC4H

The full length cDNA of RsC4H (GenBank accession No. EU123533) obtained from 3'- and 5'- RACE was 1,780 bp. In addition, the full length cDNA of RsC4H had a 1,515 bp open reading frame (ORF, including stop codon TAA), a 66 bp leader sequence (5' UTR) and a 199 bp 3' UTR. The G + C content of the ORF was 53%, those of the non-coding regions were 63% and 27% for the 5' and 3' UTR, respectively (Fig. 1A). A start codon (A67TG69) corresponded to the predicted translation start site for eukaryotic genes (A/GXXATGG) reported by Kozak (23). Also, the location of CpG islands has been reported at various positions within the genes of humans and plants. The CpG island generally exists near the 5'-end of its associated plant gene and is involved in broad expression of the gene (24, 25). In RsC4H, a 723 bp CpG island was predicted at the position C10-T781 with a G + C content of 57% and Obs./Exp of 1.16 according to the new definition of a CpG island (26). The canonical polyadenylation signal sequence is generally found 10-30 bp upstream from the polyadenylation site or 25-44 bp downstream from the stop codon (27). The 3' UTR of RsC4H contained the canonical polyadenylation signal A1727ATAAG1722 (30 bp downstream from the polyadenylation) which was slightly different from the consensus sequence AATAAA. These results are in good agreement with the canonical polyadenylation signal determined for Campyloptera acuminata C4H (AY621152). The pentanucleotide motif, ATTTA, was found in the 3' UTR (1637-1677 bp) (Fig. 1A), and is believed to confer instability to the mRNA (28). The ORF of RsC4H encodes a polypeptide of 504 amino acid residues, which contain a calculated molecular weight of 57.88 kDa and isoelectric point (pI) value of 9.1.

To address the genomic DNA structure of RsC4H, we isolate the genomic DNA of RsC4H gene in a PCR experiment using specific primers; RsC4H-F and RsC4H-R, derived from the start and stop codon regions of the cDNA. A 3,257 bp of genomic RsC4H containing the start and stop codon was isolated and had a 100% identity in the coding region to the full-length cDNA sequence. Genomic DNA of RsC4H was shown to contain three exons and two introns (Fig. 1B).

The RsC4H had all the P450-featured motifs, such as the heme-binding domain F46GVGRSCPG449, the T-containing binding pocket motif A506AETT111, and the hinge motif P450PGPIPVP41 necessary for optimal orientation of the enzyme (16, 29). These motifs were distributed in five signature sub-strate recognition sites (SRS) of C4H: SRS1 (L506RTTNVFDF1FT GKKQDMVFVTY122), SRS2 (L716AQSFE YNY224), SRS4 (L290 VENINVAIETTLWS114), SRS5 (R658MAIPLLVPH177) and SRS6 (30, 31). The SRS5 and SRS6 regions and the C-terminal end of the SRS4 region are important in contacting the aromatic rings of the substrates, and the SRS1 and SRS2 regions and N-terminal end of the SRS4 are important in contacting the aliphatic regions of the substrates. SUPERFAMILY alignment (32) revealed that RsC4H belong to the Cyt P450 family. In addition, NCBI blastp indicated that RsC4H displayed several similarities to C4Hs from other plants. The deduced amino acid sequence of RsC4H was 94% identical to MdC4H1 from Malus x domestica (AY87450) and 84% identical to AtC4H from Arabidopsis thaliana (BAA24355) (Fig. 1C). In the phylogenetic analysis using most of the published genes belonging to the CYP73 subfamily (33), RsC4H was shown to be tightly sub-grouped with MdC4H1 and other C4Hs to form a highly homologous large group (Fig. S1). These results suggest that RsC4H is related to the CYP73A5 subfamily of P450s.

Southern blot analysis

Southern blot analysis was performed to estimate the number of the RsC4H genes in the whole genome. Genomic DNA was isolated and aliquots of genomic DNA (10 μg/sample) were treated with the following restriction enzymes Xba I, Eco RV, and Hind III. Only a single Hind III recognition site, positioned at intron 2, exist within the genomic RsC4H gene, (Fig. 1B), whereas no Xba I and Eco RV recognition sites were observed within the genomic RsC4H gene. When the whole genomic DNA was subjected to restriction enzyme digestion only a single band was detected in the Xba I and Eco RV digested fragment lanes. However, two bands were observed in the lanes where the genomic DNA was digested with Hind III (Fig. 2). This result suggests that there is only a single C4H gene in the entire genome of the Korean black raspberry. Like a RsC4H, Arabidopsis thaliana, pea and parsley enigmatically contain only one C4H gene (15, 17, 22, 34), but most plants contain a small family of C4H genes e.g. at least 2 in Valencia orange (35), and Lithospermum erythrorhizon (36) and 4 in Orzya sativa (37).

RT-PCR and quantitative real-time PCR analyses

The factors affecting fruit color are predominately genetically determined, though environmental factors such as nutrients,
Cinnamate-4-Hydroxylase gene in black raspberry
Myung-Hwa Baek, et al.

Fig. 2. Southern blot analysis of RsC4H in Korean black raspberry. Genomic DNA (20 μg per lane) was digested with restriction enzymes, followed by hybridization with a 1.6 kb RsC4H cDNA probe. X, Xba I; EV, Eco RV and H, Hind III.

Fig. 3. Expression of specific RsC4H transcript during Korean black raspberry development. (A) RT-PCR analysis. The experiment was performed using total RNA isolated from different fruits at different developmental stages as described in Materials and methods. HisH3 (Rubus sp. Histone H3 gene, GeneBank accession no. AF304365) was amplified as internal controls. After 30 cycles of amplification, the PCR products were resolved in a 1.2% agarose/EtBr gel. (B) Application of real-time PCR for comparative analysis of RsC4H transcript during Korean black raspberry development. Real-time PCR was carried out in five repetitions.

Temperature and light conditions can cause a change in the flavonoid composition of the fruit. The structural or regulatory genes involved in phenylpropanoid metabolism were studied in several fruit plants, including bilberry (38-40), red raspberry (8), citrus (42), and grape (43, 44), but this has yet to be examined in the Korean black raspberry. Therefore, we investigated the spatial and temporal expression patterns of the Korean black raspberry RsC4H gene during fruit development. The expression of RsC4H gene was observed throughout ripening, but at the yellow stage of development expression was drastically reduced relative to the other color stages (Fig. 3A). Namely, the RsC4H gene during fruit development of Korean black raspberry was expressed at the beginning of fruit development and again in the late stages of ripening. In addition, we carried out SYBR Green-based quantitative real-time PCR for the detection and comparative quantification of RsC4H gene expression during fruit development. The expression level of RsC4H was calculated relative to the expression levels in green fruit as follows: ΔΔCT (Y, R, and B - G) = ΔCT (Yellow, Red and Black fruit, respectively) - ΔCT (Green fruit). From this comparative analysis the lowest relative amplification of the RsC4H gene occurred in yellow fruit. The expression levels in red and black fruit were 2 times higher relative to that of the green fruit (Fig. 3B).

These results were in accordance with the result obtained through RT-PCR analysis. These combined results indicate that the RsC4H gene in the Korean black raspberry fruit has two peaks in expression, one during the green fruit stage and a second when the fruits were nearly ripe. This pattern seems to coincide with the PAL and DFR activity peaks previously reported in strawberry fruit (45, 46).

Analysis of the content of flavanols and anthocyanins in the different developmental stages of Korean black raspberry fruit
To further investigate the ramifications of the two peaks in expression observed during fruit development and ripening, flavonoid content was also measured as a function of fruit development and ripening. Flavonoids, which are usually regarded as dispensable phytochemicals derived from plant secondary metabolism, are one of the main determinants of flower and fruit color in plants and play significant roles in the physiological activities of plants by affecting several developmental processes (5, 6, 8). Thus, the content of flavanols and anthocyanins were determined in all samples of Korean black raspberry fruits. The content of the major flavanol of Korean black raspberry fruits was highest at the beginning of fruit development and then gradually decreased according to the developmental stages (Fig. 4). Flavanols were the major flavonoids in the early developmental stages of Korean black raspberry fruit. In contrast, the content of anthocyanins during the progress of ripening dramatically increased. In fully ripened black fruits, the highest content of anthocyanins was observed. The anthocyanin content of red fruit was over 30 times higher than that of the green and yellow fruits. In addition, the content of anthocyanin at black fruit was 10 times higher than that of the red fruit and 300 times higher than that of the green and yellow fruits. These results were similar a previous report by
were harvested.

subjective assessment of color (green, yellow, red, and black),
raspberry fruit materials at four ripening stages, determined by
that were collected in late May to June, 2006. Korean black

different developmental stages were pooled from multiple plants
were pooled from several plants in late May, 2005. Fruits at dif-
stored at -80°C until used. Leaf tissues of Korean black raspberry

Center, Gochang, Republic of Korea, under ambient conditions.

Experimental plots at the Korean Black Raspberry

MATERIALS AND METHODS

Plant growth conditions and materials
Korean black raspberry (Rubus sp.) plants were grown in ex-
perimental plots at the Korean Black Raspberry Research
Center, Gochang, Republic of Korea, under ambient conditions.
All samples were immediately frozen in liquid nitrogen and
stored at -80°C until used. Leaf tissues of Korean black raspberry
were pooled from several plants in late May, 2005. Fruits at dif-
derent developmental stages were pooled from multiple plants
that were collected in late May to June, 2006. Korean black
raspberry fruit materials at four ripening stages, determined by
subjective assessment of color (green, yellow, red, and black),
were harvested.

Cloning of C4H cDNAs
Total RNA from leaves was isolated using a cetyltrimethylam-
monium bromide (CTAB) based method (47). One microgram
of total RNA was subjected to reverse-transcription (AccuPower
RT Premix, Bioneer; Daejeon, Republic of Korea) using anched
oligo (dT)16 primers. Polymerase chain reaction (PCR) using,
5'-GAAAGGAGAAGACAGCATGTTG-3' and 5'-CGTGATCT
CTGTGGATCTCT-3', degenerate primers designed from ho-
mologous sequences found in GenBank was conducted to am-
plify a region of the RsC4H gene. Based on sequence data from
this region, the 5'-terminal region of the cDNA PCR product
was amplified using the 5'-rapid amplification of cDNA ends
(RACE) method (5-Full RACE Core Set, Takara; Shiga, Japan).
A phosphorylated primer, 5'-TTCTTTTCTCTCATCAAGGA-3',
was used for the synthesis of cDNA from total RNA. The following
two primer sets were used for the first and second PCR in the
5'-RACE, respectively: a set of 5'-AGG

ACAGGACATGGTTTC-3' and 5'-GATGTCGAAGACCACCC

TTG-3' and another set of 5'-GACAGATTGTCAGCATCAGT-3'
and 5'-CCATACGGAGCATGACAC-3'.

3'-RACE was performed using a gene-specific primer, 5'-CG

ACATCTTCACCGAAAAGACACCATG-3', according to

the SMART™ (Clontech Laboratories, CA, USA) protocol. RACE
products and subsequent full-length clones were cloned into
pGEM-T Easy vector (Promega; Madison, WI, USA) and sequen-
ced.

To generate the genomic DNA of RsC4H, the genomic
DNA was isolated from the leaves by a modified CTAB meth-
od (48), and then PCR was performed with the primers,
RsC4H-F (5'-catatgGATCTCCTCATGGAGAAGAC, the re-
striction endonuclease site was represented by lower case let-
ters and the start codon was boxed) and RsC4H-R (5'-ctcgagTA
TGTCCTTGGCTTTATGCTATG-3', the restriction endonu-
cluse site was represented by lower case letters and the stop
codon was boxed), under the following condition: 94°C for 30
sec, 63°C for 30 sec, and 72°C 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 sequencing.

Southern blot analysis
Genomic DNA (gDNA) was isolated from leaves using a modi-
fied CTAB method (48). Digoxigenin (DIG) probe was synthe-
sized according to the manufacturer's instructions (PCR DIG
Probe Synthesis Kit, Roche; Molecular Biochemicals, Indiana-
polis, Indiana, USA). The gDNA was digested with Xba I, Eco
RV and Hind III, separated on a 0.7% (w/v) agarose gel, and
transferred to a positively charged blotting membrane (Zeta
Probe, Bio-Rad; Munich, Federal Republic of Germany) ac-

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Southern blot analysis
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RV and Hind III, separated on a 0.7% (w/v) agarose gel, and
transferred to a positively charged blotting membrane (Zeta
Probe, Bio-Rad; Munich, Federal Republic of Germany) ac-

 accordance to the manufacturer’s protocol. Hybridization was car-

Fig. 4. Analysis of the content of anthocyanins and flavanols in the
different developmental stages of fruits. Anthocyanin content (open-
circle) was determined by calculating A530 - 0.33A657. The content o
anthocyanin in green fruit was set to 1. r.u. = relative unit. Total fla
vanols content (closed circle) was determined as described in
Materials and methods.

(39). At the early stage of bilberry development, procyanidins
and quercetin were the predominant flavonoids, but these lev-
els decreased dramatically during the ripening progress.
During the later stage of ripening, the content of anthocyanins
was highly increased and anthocyanins were determined to be
the major flavonoids in fully ripened fruits.

Our results suggest that the early expression of RsC4H gene
may be associated with the synthesis of flavanols in green
fruits, and also it may play a role in the biosynthesis of an-
thocyanin during color development during the late stages of fruit
 ripening. This report will prove valuable for be future studies
that investigate the role of the C4H in the phenylpropanoid
metabolism in Korean black raspberry.
ried out at 50°C for overnight in a DIG Easy Hyb (Roche; Mannheim, Germany). After stringent washing (with 2X SSC, 0.1% SDS (w/v) twice at room temperature for 5 min, then with 0.1X SSC, 0.1% SDS twice at 68°C for 15 min under constant agitation), bands were visualized using a DIG Nucleic Acid Detection Kit (Roche; Mannheim, Germany).

Reverse transcription (RT)-PCR

Total RNA from fruit samples was isolated using a CTAB based method (47). The first strand of the cDNA was synthesized using anchored oligo (dT)$_{25}$V primers as described above. PCR reaction was performed using 5'-GTTGTCCTCGATCTTGATCTCTC-3' and 5'-GTGGTCTTCGACATCTTAG-3', oligonucleotide primers directed at the 389 and 908 basepairs of the RsC4H cDNA. PCR conditions were as follows: 94°C for 5 min, followed by 30 cycles of 94°C for 45 sec, 52°C for 45 sec, 72°C for 45 sec, and completed with 72°C for 10 min. The control primers used to amplify the Rubus sp. Histone H3 gene and PCR conditions were described by Kumar and Ellis (8).

Real time-PCR

Specific primers for RsC4H, 5'-GAGGCTTCAGTACAATAGC-3' and 5'-GATCTCTCTCTTTCTGAG-3' were used to perform real time-PCR. The SYBR Green real-time PCR assay was performed using Premix Ex Taq$^\text{TM}$ (Takara, Shiga, Japan), 0.2 μM (each) specific primers and 1 μl template cDNA. The amplification program consisted of one cycle at 94°C for 60 sec, followed 45 cycles of 94°C for 5 sec, 45°C for 45 sec, and 72°C for 15 sec. The fluorescent product was detected at the last step of each cycle. After amplification, melting temperatures of the PCR products were analyzed to determine the specificity of the PCR. Melting curves were obtained by slow heating at 0.2°C/sec from 60°C to 90°C while continuously monitoring the fluorescence signal. Amplification, acquisition, and analysis were carried out with Smart Cycler$^\text{II}$ System (Cepheid, Sunnyvale, CA, USA) and Smart Cycler software (version 2.0 c.), which determines the threshold cycle (Ct). Ct represents the number of cycles at which the fluorescence intensity was significantly higher than the background fluorescence at the initial exponential phase of PCR amplification. To determine the relative fold differences in template abundance for each sample the Ct value for RsF3H was normalized to the Ct value for Histone H3 and calculated relative to a calibrator using the formula $2^{-\Delta \Delta Ct}$ (49).

Analysis of the content of anthocyanin and flavanols

Samples (0.1 g) were extracted overnight at 4°C in 300 μl of the extraction buffer (7% HCl in methanol). A 200 μl volume of water was added to the extract, mixed, and centrifuged at 20,000 g for 10 min. A 600 μl volume of 1% HCl in methanol was then added to 400 μl of the supernatant and centrifuged at 20,000 x g for 10 min. Anthocyanin analysis was performed using a previously described protocol (50-52). Anthocyanin content was determined by calculating $A_{330}$ - 0.33$A_{520}$. For the identification of flavanols, a Shimadzu HPLC system (SPD-20A) equipped with YMC gel ODS A302 (4.6 mm i.d x 150 mm) was used. Flavanols were monitored by absorbance at 360 nm using Shimadzu SPD-20A UV-VIS detector. The total flavanol content was determined by the vanillin-HCl method as described previously (53). The vanillin assays in methanol were carried out in a 30°C water bath with a reaction time of 20 min. The vanillin reagent contained 4% HCl and 0.5% vanillin in methanol. At time zero 1 ml of the sample was added to 5 ml of the reagent solution and mixed. After 20 min, the absorbance was recorded at 500 nm. Catechin was used as a standard for this assay.

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