Accumulation of Tilianin and Rosmarinic Acid and Expression of Phenylpropanoid Biosynthetic Genes in Agastache rugosa

Pham Anh Tuan†,‡ Woo Tae Park†,‡ Hui Xu,† Nam Il Park§, and Sang Un Park⁎†

†Department of Crop Science, College of Agriculture & Life Sciences, Chungnam National University, 220 Gung-dong, Yuseong-gu, Daejeon 305-764, Korea
‡Wildlife Genetic Resources Center, National Institute of Biological Resources (NIBR), Gyungseo-dong, Seo-gu, Incheon 404-708, Korea

Supporting Information

ABSTRACT: Korean mint (Agastache rugosa), a perennial, medicinal plant of the Labiatae family, has many useful constituents, including monoterpenes and phenylpropanoids. Among these, tiliacin and rosmarinic acid, 2 well-known natural products, have many pharmacologically useful properties. Chalcone synthase (CHS) and chalcone isomerase (CHI) catalyze the first and second committed steps in the phenylpropanoid pathway of plants, leading to the production of tiliacin. In this study, cDNAs encoding CHS (ArCHS) and CHI (ArCHI) were isolated from A. rugosa using rapid amplification of cDNA ends (RACE)-PCR. Amino acid sequence alignments showed that ArCHS and ArCHI shared high sequence identity and active sites with their respective orthologous genes. Quantitative real-time PCR analysis was used to determine the expression levels of genes involved in tiliacin and rosmarinic acid biosyntheses in the flowers, leaves, stems, and roots of A. rugosa. High-performance liquid chromatography (HPLC) revealed that the accumulation pattern of tiliacin matched the expression patterns of ArCHS and ArCHI in different organs of A. rugosa. Moreover, acacetin, the precursor of tiliacin, also demonstrated an accumulation pattern congruent with the expression of these 2 genes. The transcription levels of ArPAL, ArC4H, and Ar4CL were the highest in the leaves or flowers of the plant, which also contained a relatively high amount of rosmarinic acid. However, the roots showed a significant content of rosmarinic acid, although the transcription of ArPAL, ArC4H, and Ar4CL were low. The findings of our study support the medicinal usefulness of A. rugosa and indicate targets for increasing tiliacin and rosmarinic acid production in this plant.

KEYWORDS: Agastache rugosa, gene expression, phenylpropanoid, rosmarinic acid, tiliacin

INTRODUCTION

Agastache rugosa, belonging to the mint family (Labiatae), is a perennial medicinal plant widely distributed throughout East Asian countries. In Korea, this plant is a ubiquitous herb and has been used as a wild vegetable and an herbal drug in traditional therapies. Studies have indicated that A. rugosa has a variety of pharmacological activities such as anti-HIV integration activities and antifungal effects. Extracts of A. rugosa are also believed to be valuable in the treatment of inflammatory and oxidative stress-induced disorders.

A. rugosa contains several types of essential oils, sesquiterpenes, diterpenes, triterpenes, flavonoids, and carotenoids. Among these, tiliacin, which is considered to be the main flavonoid, and rosmarinic acid have been proven to contribute to the medicinal potential of A. rugosa. Tiliacin is found in various plants as a glucose-glycoside compound of a flavonoid, acacetin. Natural product chemists and clinicians have been interested in tiliacin because of its important biological activities, for example, its anti-inflammatory, antiatherogenic, antihypertensive, and vasorelaxant effects. Rosmarinic acid, a well-known hydroxycinnamic acid ester, was identified as an active component in several medicinal plants. The biosynthesis and production of rosmarinic acid in plants have been extensively studied, because it exhibits various pharmacological properties, including antiviral, antibacterial, anti-inflammatory, and antioxidant effects. In the plant kingdom, rosmarinic acid is thought to act as a preformed, constitutively accumulated defense compound.

The proposed biosynthetic pathway of tiliacin and rosmarinic acid in plants is shown in Figure 1. The amino acid L-phenylalanine is the starting compound for the biosynthesis of both compounds. Phenylalanine is transformed to 4-coumaroyl-CoA by 3 enzymes in the general phenylpropanoid pathway, viz., phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H), and 4-coumarate:CoA ligase (4CL). 4-Coumaroyl-CoA is the precursor for many secondary plant products, such as flavonoids, stilbenes, coumarins, and lignin. In the next step, naringenin chalcone. Chalcone isomerase (CHI) converts naringenin chalcone to naringenin via a stereospecific isomerization. In the next step, flavone synthase (FS) catalyzes the desaturation of naringenin to derive apigenin. Then, apigenin 4′-O-methyltransferase (A4OMT) transfers a methyl group to apigenin to yield acacetin. Finally, glucosyltransferase (GT) catalyzes the transfer of glucose to acacetin to synthesize tiliacin.
acid, condensation of 4-hydroxyphenyllactic acid with 4-coumaroyl-CoA is catalyzed by hydroxycinnamoyl-CoA:hydroxyphenyllactate hydroxycinnamoyl transferase (RAS) to form 4-coumaroyl-4′-hydroxyphenyllactic acid. 4-Coumaroyl-4′-hydroxyphenyllactic acid is then converted to rosmarinic acid by 2 consecutive hydroxylation steps.21 These reactions are catalyzed by 2 distinct cytochrome P450s, viz., 3-hydroxycinnamoyl (3-H) and 3′-hydroxycinnamoyl (3′-H).22

In this study, cDNAs encoding chalcone synthase (ArCHS) and chalcone isomerase (ArCHI), which are related to tilianin biosynthesis in A. rugosa, were isolated. The expression patterns of genes involved in tilianin and rosmarinic acid biosyntheses (ArPAL, ArC4H, Ar4CL, ArCHS, and ArCHI) were analyzed by real-time PCR. The concentrations of tilianin and rosmarinic acid in different plant organs were also determined by HPLC to investigate the biosynthetic mechanisms of these compounds in A. rugosa.

MATERIALS AND METHODS

Plant Materials. A. rugosa plants were grown at the experimental farm of Chungnam National University (Daejeon, Korea). The flowers, leaves, stems, and roots were excised from mature plants. The samples were immediately frozen in liquid nitrogen and then stored at −80°C and/or freeze-dried for RNA isolation and/or HPLC analysis.

Figure 1. Proposed biosynthetic pathway of tilianin and rosmarinic acid. PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumaryl-CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase; FS, flavone synthase; A4OMT, apigenin 4′-O-methyltransferase; GT, glucosyltransferase; RAS, hydroxycinnamoyl-CoA:hydroxyphenyllactate hydroxycinnamoyl transferase; 3-H, hydroxycinnamoyl; 3′-H, hydroxycinnamoyl.
RNA Isolation and cDNA Synthesis. Total RNA was isolated from different organs of *A. rugosa* separately using Plant total RNA mini kit (Geneaid, Taiwan) under the manufacturer's instruction. The quantity and concentration of total extracted RNA were determined by 1% agarose gel electrophoresis and spectrophotometer analysis, respectively. For RACE-PCR, 3 μg of total RNA was used to synthesize first-strand cDNA using GeneRacer Kit (Invitrogen, Carlsbad, CA, USA) and a 10-fold dilution of the resulting cDNA was used as template. For quantitative real-time PCR, 1 μg of total RNA from different organs was used for reverse transcription using the ReverTra Ace-R kit (Toyobo, Osaka, Japan) and a 20-fold dilution of the resulting cDNA was used as template.

Cloning of the cDNA Encoding Chalcone Synthase and Chalcone Isomerase. Degenerate primers for *CHS* and *CHI* were designed based on homologies found in genes isolated earlier (Table 1).

### Table 1. Primers Used in This Study

<table>
<thead>
<tr>
<th>primer</th>
<th>sequence (5’ to 3’)</th>
<th>amplicon (base pairs)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>ArCHS</em>3</td>
<td>TGAAGCCCTCTGATGTTACCCAGCA</td>
<td>970</td>
</tr>
<tr>
<td><em>ArCHS</em>5</td>
<td>CTCGGACGACGGACAAAAACCTTA</td>
<td>650</td>
</tr>
<tr>
<td><em>ArCHI</em>3</td>
<td>TCCAGTTCTACTGCGGAAATCT</td>
<td>681</td>
</tr>
<tr>
<td><em>ArCHI</em>5</td>
<td>TTCTCTCAGATTTGGCTGGAGCCTG</td>
<td>426</td>
</tr>
<tr>
<td><em>ArActin_RT</em> F</td>
<td>ACCCTCAAAATGACATTGGGAAGT</td>
<td>151</td>
</tr>
<tr>
<td><em>ArActin_RT</em> R</td>
<td>GGGCCGCCTCCATCTACCTATTGCTA</td>
<td>108</td>
</tr>
<tr>
<td><em>ArPAL_RT</em> F</td>
<td>ATCCGGCCACCGGCTTAATAAT</td>
<td>157</td>
</tr>
<tr>
<td><em>ArPAL_RT</em> R</td>
<td>ATCCGGGTTTACCCTTCCTCAGGT</td>
<td>162</td>
</tr>
<tr>
<td><em>Ar4CL_RT</em> F</td>
<td>GTTCGAGATGGAAGTACCGCCTG</td>
<td>151</td>
</tr>
<tr>
<td><em>Ar4CL_RT</em> R</td>
<td>ATATCCCCTGAAACATTTCGCCGCC</td>
<td>175</td>
</tr>
<tr>
<td><em>ArPAL_RT</em> F</td>
<td>TTGGGTTCTCTTCCGATGGTACC</td>
<td>154</td>
</tr>
<tr>
<td><em>ArPAL_RT</em> R</td>
<td>GCTTCCCTAAAAGATGTTCTGT</td>
<td>194</td>
</tr>
<tr>
<td><em>ArCHI_RT</em> F</td>
<td>TCTTGATGGCCTTTTGGCGTTCAGC</td>
<td>394</td>
</tr>
<tr>
<td><em>ArCHI_RT</em> R</td>
<td>TCTTGATGCTTCTTTGCGTCAGC</td>
<td>426</td>
</tr>
</tbody>
</table>

1). The amplified products were purified and cloned into T-blunt vector (Solgent, Daejeon, Korea) followed by sequencing. BLAST results confirmed those fragments were partial of *CHS* and *CHI* from *A. rugosa*. Using the obtained partial sequences, specific gene primers was designed to amplify the 5’-end and 3’-end of *CHS* and *CHI*. 5’-RACE-PCR and 3’-RACE-PCR were performed following the manufacturer's protocol. Degenerate primers *CHS* F and *CHS* R amplified a 492-bp fragment, of which the sequence showed similarity to other CHSs according to a BLAST search. Subsequently, the 5’-end and 3’-end of the *ArCHS* were generated using RACE-PCR primers. The expected PCR product was purified and cloned into T-blunt vector for sequencing.

Sequence Analysis. The deduced amino acid sequences of *ArCHS* and *ArCHI* were analyzed for homology using BLAST at the NCBI Genbank database (http://www.ncbi.nlm.nih.gov/BLAST). Sequencing alignments were carried out by BioEdit Sequence Alignment Editor, version 5.0.9 (Department of Microbiology, North Carolina State University, Raleigh, NC). Phylogenetic tree was constructed by the online Web site (http://www.sensible.org).
observed in the phylogenetic analysis (Figure S2 in the Supporting Information).

Expression Levels of Genes Involved in Tilianin and Rosmarinic Acid Biosyntheses in Different Organs of A. rugosa. Quantitative real-time PCR analysis was used to investigate the expression patterns of ArPAL, ArC4H, Ar4CL, ArCHS, and ArCHI in the flowers, leaves, stems, and roots of A. rugosa. As shown in Figure 4, the expression of ArPAL was the highest in the leaves, where the relative quantification to actin (RQ-value) was 0.14. ArPAL was expressed at a moderate level in the flowers (RQ 0.06) and stems (RQ 0.06) and was the lowest in the roots (RQ 0.02). In contrast to this first enzyme, the transcription level of the second enzyme, ArC4H, was the most abundant in the flowers (RQ 30.52), lower in the leaves (RQ 11.87) in the flowers, which was expressed at a moderate level in the leaves (RQ 11.9) and the lowest in the root (RQ 2.14). The level of Ar4CL was the highest in the leaves, with an RQ of 17.98, higher than the RQ of 11.87 in the flower. A lower expression of Ar4CL was found in the stems (RQ 8.66) and roots (RQ 5.45). ArCHS transcription was abundant in the flowers (RQ 2.9), intermediate in the leaves (RQ 1.28), and poor in the stems (RQ 0.2) and roots (RQ 0.18). The expression pattern of ArCHI was fairly similar to that of ArCHS, with the highest expression in the flowers (RQ 4.28), moderate expression in the leaves (RQ 1.24), and weak expression in the stems (RQ 0.26) and roots (RQ 0.16).

Analysis of Rosmarinic Acid and Tilianin Contents in Different Organs of A. rugosa. The same plant materials as those used for quantitative real-time PCR were used for HPLC analysis of tilianin and rosmarinic acid in A. rugosa. Tilianin, its precursor acacetin, and rosmarinic acid were measured in the
flowers, leaves, stems, and roots of *A. rugosa* (Figure 5). Accumulation of acacetin was the highest in the flower, where its concentration was 0.84 μg/g of dry weight. The concentration of acacetin was very low in the leaves (0.06 μg/g) and was undetectable in the stems and roots. Moreover, the stems and roots also contained only a miniscule amount of tilianin, viz., 0.49 μg/g and 0.14 μg/g, respectively. However, tilianin levels in the leaves and flowers were markedly higher than acacetin levels. Tilianin content in the leaves was 2.18 μg/g, while an appreciable content, 6.33 μg/g, was found in the flowers. The accumulation patterns of acacetin and tilianin correlated with the expression patterns of *ArCHS* and *ArCHI*, as shown in Figure 4.

Compared to acacetin and tilianin, rosmarinic acid was more abundantly synthesized in *A. rugosa*. Specifically, levels of rosmarinic acid were the highest in the flowers (48.43 μg/g), significant in the roots (30.97 μg/g) and leaves (22.14 μg/g), and the lowest in the stems (9.14 μg/g).

Although tilianin and rosmarinic acid have been detected and studied for a long time in *A. rugosa*, there are no reports on their biosyntheses in this plant. In the present study, CHS and CHI, which relate to the formation of tilianin, were first cloned.
in A. rugosa. Transcription of genes involved in tilianin and rosmarinic acid biosynthetic pathways (ArPAL, ArC4H, Ar4CL, ArCHS, and ArCHI) were analyzed in different organs of A. rugosa. They were expressed constitutively with the highest levels in the flowers or leaves and the lowest levels in the roots. The expression of PAL, C4H, and 4CL were previously found to correlate with the flavonoid contents in various plants. The high transcription levels of ArPAL, ArC4H, and Ar4CL may explain the high levels of the flavonoid, acacetin, and its derivative, tilianin, in the flowers and leaves of A. rugosa (Figures 4 and 5). Similarly, the low expression levels of ArPAL, ArC4H, and Ar4CL may be responsible for the trace amounts of acacetin and tilianin detected in the roots.

The pattern of expression of ArCHS and ArCHI matched the accumulation patterns of acacetin and tilianin in different organs of A. rugosa conspicuously. CHS and CHI catalyze the first and the second committed steps in the phenylpropanoid pathway of plants, leading to acacetin, tilianin, and other flavonoids. Earlier studies found that CHS and CHI are the rate-limiting enzymes of the flavonoid biosynthetic pathway in some plants. This indicates that ArCHS and ArCHI may regulate the biosyntheses of acacetin and tilianin as well as other flavonoids in A. rugosa.

Three enzymes of the general phenylpropanoid pathway (PAL, C4H, and 4CL) have been proven to be important for rosmarinic acid biosynthesis in some plants. In Salvia miltiorrhiza, PAL was demonstrated to be a key enzyme in the rosmarinic acid biosynthetic pathway; the fluctuation of rosmarinic acid content directly correlated with PAL expression. Moreover, the downregulation of PAL affected the expression of C4H and 4CL and caused a reduction in rosmarinic acid content in S. miltiorrhiza. The expression patterns of PAL and 4CL correlated with the enzyme activities and with the rosmarinic acid content of Melissa officinalis in a suspension culture. In A. rugosa, 3 enzymes of the general phenylpropanoid pathway had the highest expression in the leaves (ArPAL and Ar4CL) or flowers (ArC4H), which also contained a relatively high content of rosmarinic acid. However, the roots showed a significant content of rosmarinic acid, although the transcription levels of ArPAL, ArC4H, and Ar4CL were low. These results contrast with previous results found in S. miltiorrhiza, in which the expression of PAL was abundant in the roots, which had a low content of rosmarinic acid. This suggests that the biosynthesis of rosmarinic acid in plants is controlled by a complex mechanism.

The total concentration of tilianin was considerably higher than that of acacetin in A. rugosa. This is in accordance with a previous study in which tilianin was the major flavonoid in A. rugosa. Furthermore, tilianin and acacetin were distributed mostly in the flowers and leaves, the organs of maximum interest in A. rugosa plants. The high amounts of tilianin and acacetin in flowers and leaves would result from a combination of endogenous biosynthesis, on one hand, and transport from the stems and roots, where only small amounts were detected, on the other. Rosmarinic acid, which is a potentially useful medicinal compound, was found abundantly in the flowers (48.43 mg/g) and roots (30.97 mg/g) of A. rugosa. This indicates that A. rugosa might be a valuable medicinal plant. In conclusion, the sequences of CHS and CHI, together with the tilianin and rosmarinic acid analyses, may broaden our understanding of the molecular mechanisms involved in their biosynthetic pathways occurring in A. rugosa. Moreover, our study also indicates targets for increasing the production of tilianin and rosmarinic acid in A. rugosa.

**ASSOCIATED CONTENT**

**Supporting Information**

Two figures showing phylogenic tree of ArCHS and ArCHI and some of their homologues. This material is available free of charge via the Internet at http://pubs.acs.org.

**AUTHOR INFORMATION**

*Corresponding Author*

Department of Crop Science, College of Agriculture & Life Sciences, Chungnam National University, 220 Gung-dong, Yuseong-gu, Daejeon, 305-764, Korea. Phone: +82-42-821-5730. Fax: +82-42-822-2631. E-mail: supark@cnu.ac.kr.

**Author Contributions**

‡These authors contributed equally to the paper.

**Funding**

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (20110010231).

**Notes**

The authors declare no competing financial interest.

**ABBREVIATIONS USED**

DEPC, diethylpyrocarbonate; HPLC, high-performance liquid chromatography; RACE, rapid amplification of cDNA ends; PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumaryl-CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase; FS, flavone synthase; A4OMT, apigenin 4’-O-methyltransferase; GT, glucosyltransferase; RAS, hydroxycinnamoyl-CoA:hydroxyphenyllactate hydroxycinnamoyl transferase; 3-H, 3-hydroxycinnamoyl; 3’-H, 3’-hydroxycinnamoyl

**REFERENCES**


(28) Singh, K.; Kumar, S.; Rani, A.; Gulati, A.; Ahuja, P. Phenylalanine ammonia-lyase (PAL) and cinnamate 4-hydroxylase (C4H) and catechins (flavan-3-ols) accumulation in tea. *Funct. Integr. Genomics* 2009, 9, 125–134.