J. Microbiol. Biotechnol. (2010), **20**(9), 1295–1299
doi: 10.4014/jmb.1005.05038
First published online 24 July 2010

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Biosynthesis of Plant-Specific Flavones and Flavonols in *Streptomyces venezuelae*

Park, Sung Ryeol†, Ji Hye Paik†, Mi Sun Ahn, Je Won Park, and Yeo Joon Yoon*

Department of Chemistry and Nano Science, Ewha Womans University, Seoul 120-750, Korea

Received: May 27, 2010 / Accepted: June 8, 2010

Recently, recombinant *Streptomyces venezuelae* has been established as a heterologous host for microbial production of flavanones and stilbenes, a class of plant-specific polyketides. In the present work, we expanded the applicability of the *S. venezuelae* system to the production of more diverse plant polyketides including flavones and flavonols. A plasmid with the synthetic codon-optimized flavone synthase I gene from *Petroselium crispum* was introduced to *S. venezuelae* DHS2001 bearing a deletion of the native pikromycin polyketide synthase gene, and the resulting strain generated flavones from exogenously fed flavanones. In addition, a recombinant *S. venezuelae* mutant expressing a codon-optimized flavanone 3β-hydroxylase gene from *Citrus siensis* and a flavonol synthase gene from *Citrus unshius* also successfully produced flavonols.

Keywords: Flavones, flavonol, heterologous production, *Streptomyces venezuelae*

Phenylpropanoids including flavanones, flavones, flavonols, and stilbenes are plant secondary metabolites that have attracted much attention as pharmaceuticals and nutraceuticals because of their antitumor, antioxidant, anti-inflammatory, and antibacterial activities [1, 12, 13]. They are biosynthesized through the phenylpropanoid pathway, in which phenylpropanoic acids are used to generate flavanones by sequential reaction of 4-coumarate/cinnamate:coenzyme A ligase (4CL), chalcone synthase (CHS), and chalcone isomerase (CHI). These flavanones are further modified into flavones and flavonols by flavone synthase (FNS) and the flavanone 3β-hydroxylase (F3H)–flavonol synthase (FLS) pair, respectively, as shown in Fig. 1 [12]. Stilbene synthase (STS) acts on the same substrate as CHS, but the ring folding in the condensation reaction differs, which leads to the production of stilbenes [11]. Recently, we demonstrated that the heterologous expression of synthetic codon-optimized flavanone and stilbene biosynthetic genes in *Streptomyces venezuelae* DHS2001 [3] supplemented with phenylpropanoic acid substrates (4-coumaric acid and cinnamic acid) resulted in the production of flavanones (naringenin and pinocembrin, respectively) and stilbenes (resveratrol and pinosylvin, respectively) [9].

In this study, a plasmid expressing the codon-optimized flavone synthase I gene from *Petroselium crispum* ($pcFNS_{op}$) under the control of the *ermE** promoter (P_{ermE*}) was constructed and introduced into the recombinant *S. venezuelae* DHS2001. The resulting strain produced apigenin and chrysin from naringenin and pinocembrin, respectively. Feeding *S. venezuelae* DHS2001 expressing the codon-optimized flavanone 3β-hydroxylase gene and flavonol synthase gene from *Citrus siensis* ($csF3H_{op}$) and *Citrus unshius* ($cuFLS_{op}$), respectively, with flavanone substrates led to the production of the flavonols kaempferol and galangin.

The high-copy number *Escherichia coli*–*Streptomyces* shuttle vector pSE34 containing a strong *ermE** promoter (P_{ermE*}) plus a thiostrepton resistance marker was used as an expression plasmid. The DNA fragments $pcFNS_{op}$ (GenBank Accession No. AY230247), $csF3H_{op}$ (GenBank Accession No. AB011795), and $cuFLS_{op}$ (GenBank Accession No. AB011796) with restriction sites and ribosome binding site (RBS) AGGAGG were designed to optimize the codon usages of them for *S. venezuelae* [2] and synthesized by Genotech, Inc. (Daejon, Korea). Each DNA fragment contained an *Xba*I site upstream of the RBS and an *Spe*I–*Hin*dIII site downstream of the stop codon to facilitate subcloning. Plasmids pYJ858 (Fig. 2A) containing P_{ermE*}-$pcFNS_{op}$ and pYJ861 (Fig. 2B) containing P_{ermE*}-$csF3H_{op}$-P_{ermE*}-$cuFLS_{op}$ were constructed as previously described [9] and introduced into the *S. venezuelae* DHS2001 [3]. For production of flavones and flavonols, an engineered *S. venezuelae* DHS2001 harboring pYJ858 (DHS2001/pYJ858) or pYJ861 (DHS2001/pYJ861) was cultivated at 30°C for 66 h in 5 ml of R2YE liquid medium [4] supplemented with 0.5 mM naringenin or pinocembrin. The cultures of *S. venezuelae* DHS2001/pYJ858 and DHS2001/pYJ861

*Corresponding author
Phone: +82-2-3277-4082; Fax: +82-2-3277-3419;
E-mail: joonyoon@ewha.ac.kr

†Park and Paik contributed equally to this work

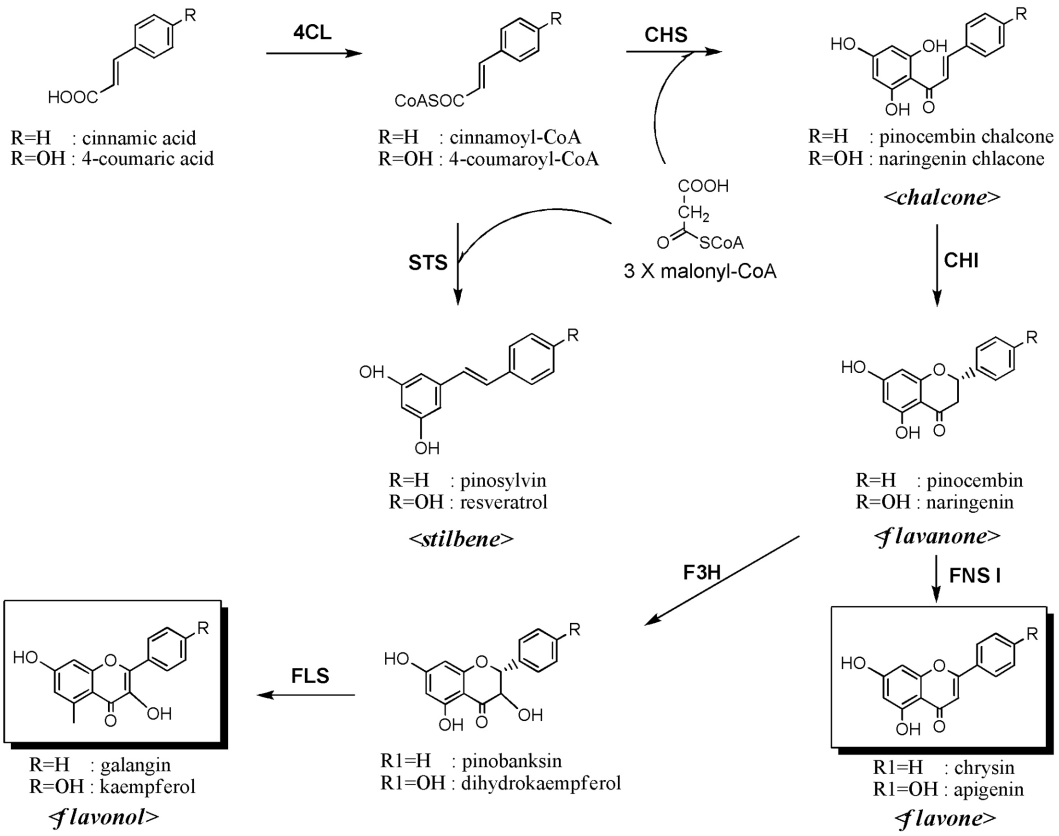

Fig. 1. Engineered flavonoid biosynthetic pathway in *Streptomyces venezuelae*.
Naringenin and pinocembrin were supplemented into the culture broth of the recombinant *S. venezuelae* DHS2001/pYJ858 and DHS2001/pYJ861. Enzyme abbreviations: 4CL, 4-coumarate/cinnamate:coenzyme A ligase; CHS, chalcone synthase; CHI, chalcone isomerase; STS, stilbene synthase; FNS I, flavone synthase I; F3H, flavanone 3β-hydroxylase; FLS, flavonol synthase.

were extracted with 1 volume of ethyl acetate and analyzed by liquid chromatography–electrospray ionization-mass spectrometry (LC–ESI–MS/MS, Waters/Micromass Quattro *micro*/MS), which was conducted using a Phenomenex Synergi Polar-RP column (150×4.6 mm, 4 μm) in the positive-ion mode. Quantification was conducted in multiple reactions monitoring (MRM) mode in MS/MS. To accomplish this, two mass ion sets were selected to detect the following transitions of the parent ions to the product ions specific to the selected analytes: apigenin and galangin 271>153; chrysin 255>153; kaempferol 287>153. Apigenin, chrysin, kaempferol, and galangin, which were used as authentic standards, were purchased from Sigma-Aldrich.

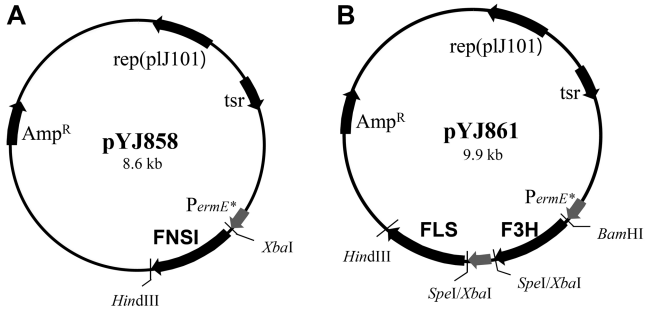

Fig. 2. Expression plasmids carrying flavone and flavonol biosynthetic genes.
A. pYJ858 containing $pcFNS_{op}$. **B**. pYJ861 containing $csF3H_{op}$ and $cuFLS_{op}$. Gene abbreviation: $pcFNS_{op}$, $csF3H_{op}$, and $cuFLS_{op}$: codon-optimized genes encoding flavone synthase I derived from *Petroselium crispum*, flavanone 3β-hydroxylase from *Citrus siensis*, and flavonol synthase from *Citrus unshius*, respectively.

LC–ESI–MS/MS analysis showed that 1.4 mg/l of apigenin and 2.9 mg/l of chrysin were produced by *S. venezuelae* DHS2001/pYJ858 supplemented with 0.5 mM naringenin and pinocembrin, respectively (Fig. 3C, 3D, 3E, and 3F), whereas no flavones were detected in the cultures of *S. venezuelae* DHS2001 harboring empty plasmid (data not shown). The metabolite eluted at 20.9 (Fig. 3C) and 28.4 min (Fig. 3D) were found to have a molecular ion $[M+H]^+$ at *m/z* 271 and 255, with typical fragments at *m/z* 153, respectively, which can be interpreted as apigenin and chrysin, respectively (Fig. 3E and 3F). Apigenin and chrysin standards showed smilar retention times (Fig. 3A and 3B) and MS/MS fragmentations (data not shown).

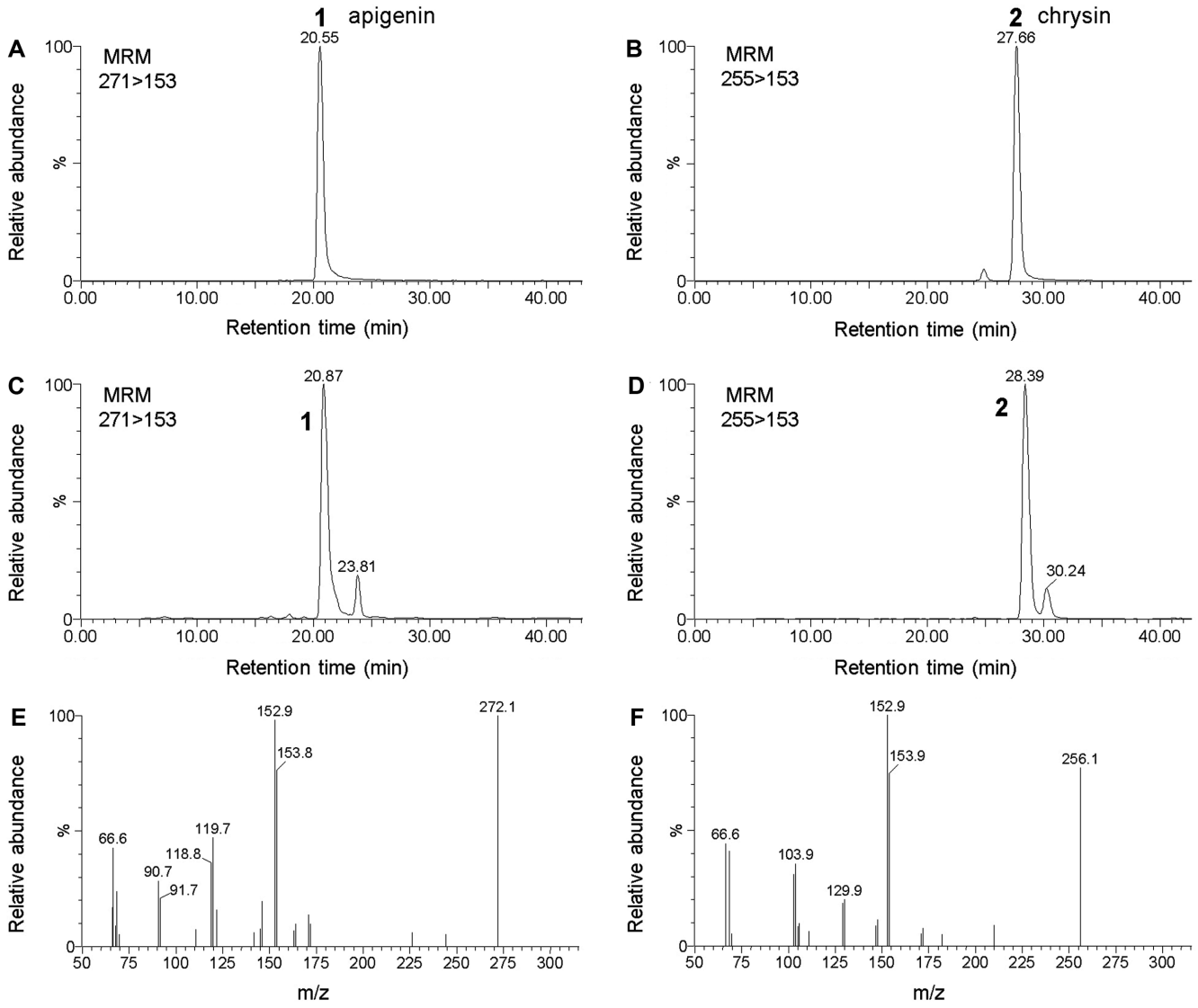

Fig. 3. HPLC–ESI–MS and ESI–MS/MS analyses of flavones obtained from *S. venezuelae* DHS2001/pYJ858.
LC–ESI–MS chromatograms of (**A**) standard apigenin (peak 1), (**B**) chrysin (peak 2), (**C** and **D**) extracts obtained from *S. venezuelae* DHS2001/pYJ858 supplemented with naringenin and pinocembrin, respectively, and (**E** and **F**) apigenin and chrysin produced by *S. venezuelae* DHS2001/pYJ858, respectively.

In addition, the genes required for flavonol biosynthesis, synthetic codon-optimized *C. siensis* F3H ($csF3H_{op}$) and *C. unshius* FLS ($cuFLS_{op}$), were cloned into pSE34 to construct pYJ861 (Fig. 2B). When 0.5 mM naringenin and pinocembrin were used as precursors, the recombinant strain of *S. venezuelae* expressing this plasmid (DHS2001/pYJ861) produced kaempferol (0.2 mg/l) and galangin (1.0 mg/l), respectively, as verified by LC–ESI–MS/MS (Fig. 4C, 4D, 4E, and 4F). However, no kaempferol and galangin were detected in the culture of *S. venezuelae* DHS2001 harboring empty plasmid (data not shown). The metabolite eluted at 28.4 (Fig. 4C) and 21.4 min (Fig. 4D) showed a molecular ion $[M+H]^+$ at *m/z* 287 and 271 with typical fragments at *m/z* 153, respectively. Based on a comparison of the retention time and MS/MS spectral data with those of authentic kaempferol and galangin (Fig. 4E and 4F), the compounds produced by *S. venezuelae* DHS2001/pYJ861 were identified as kaempferol and galangin.

Here, the flavonoid biosynthetic genes were expressed in an engineered strain of *S. venezuelae*, which led to the production of flavones and flavonols. This result expanded the applicability of *S. venezuelae* as a heterologous host for *in vivo* production of a diverse class of plant polyketides. Although the current productivities of flavones and flavonols in *S. venezuelae* are relatively low when compared with that of another heterologous host, *E. coli* [9], there is still room for productivity improvement. Recently, Miyahisa *et al*. [8] accomplished metabolic engineering directed at

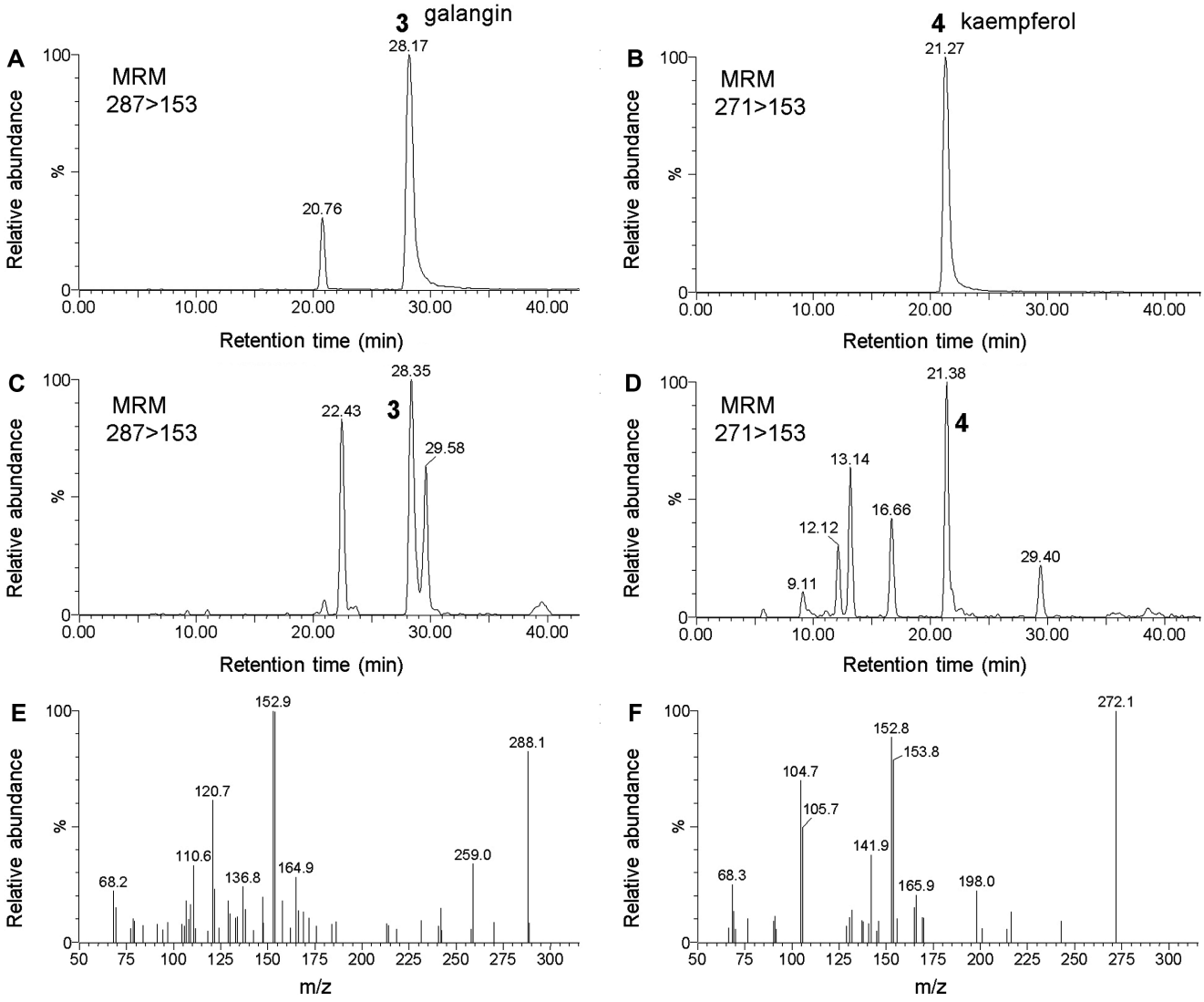

Fig. 4. HPLC–ESI–MS and ESI–MS/MS analyses of flavonols obtained from *S. venezuelae* DHS2001/pYJ861.
LC–ESI–MS chromatogram of (**A**) standard kaempferol (peak 3), (**B**) galangin (peak 4), (**C** and **D**) extracts obtained from *S. venezuelae* DHS2001/pYJ861 supplemented with naringenin and pinocembrin, respectively, and (**E** and **F**) kaempferol and galangin produced by *S. venezuelae* DHS2001/pYJ861, respectively.

increasing carbon flux toward malonyl-CoA that led to a significant increase in the flavanone production in *E. coli* (approximately 3-folds). Furthermore, another study reported that introduction of *matB* and *matC* genes encoding malonate synthetase and malonate carrier protein from *Rhizobium triofoli*, respectively, into the recombinant *E. coli* expressing *4CL*, *CHS*, and *CHI* resulted in the production of approximately 155 mg/l of flavanones [6]. Thus, we expect that metabolic engineering of *S. venezuelae* to increase the intracellular malonyl-CoA level will lead to further enhancement of the yields.

On the other hand, *O*-methyltransferase from *Streptomyces avermitilis* was successfully used in the biotransformation of naringenin to antifungal sakuranetin [5]. Similarly, substrate-flexible *O*-methyltransferases from *S. venezuelae* might enable the biosynthesis of more structurally diverse phenylpropanoids. In addition, the more valuable characteristics such as rapid growth and relative ease of genetic manipulation of *S. venezuelae*, compared with other actinomycete heterologous hosts, as a well as well-developed multigene expression system [3, 10] will provide another advantage for the production of a diverse class of plant-derived polyketides.

Acknowledgments

This work was supported by the Korea Research Foundation Grant funded by the Korean Government (MOEHRD, Basic Research Promotion Fund) (KRF-2007-313-D00250).

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