

Enhanced Flavonoid Production in *Streptomyces venezuelae* via Metabolic Engineering

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Metabolic engineering of plant-specific phenylpropanoid biosynthesis has attracted an increasing amount of attention recently, owing to the vast potential of flavonoids as nutraceuticals and pharmaceuticals. Recently, we have developed a recombinant *Streptomyces venezuelae* as a heterologous host for the production of flavonoids. In this study, we successfully improved flavonoid production by expressing two sets of genes predicted to be involved in malonate assimilation. The introduction of *matB* and *matC* encoding for malonyl-CoA synthetase and the putative dicarboxylate carrier protein, respectively, from *Streptomyces coelicolor* into the recombinant *S. venezuelae* strains expressing flavanone and flavone biosynthetic genes resulted in enhanced production of both flavonoids.

Keywords: Flavonoids, metabolic engineering, biosynthesis, *Streptomyces venezuelae*

Flavonoids, a highly diverse class of plant secondary metabolites, carry out a broad range of important functions in plants, including roles as pigments, protectants against stresses, structural components, and signaling molecules [9, 11]. Recent studies evaluating the health-promoting effects of these plant-derived natural products have generally highlighted their potential in pharmaceutical and nutraceutical applications; however, their limited availability due to seasonal and regional variations and the difficulty inherent to the isolation of single compounds from plants remain intrinsic challenges in this regard [9, 12]. As a consequence, the development of efficient microbial processes has emerged as a possible practical alternative for large-scale production. As shown in Fig. 1, the conversion of phenylpropanoic acids such as 4-coumaric acid and cinnamic acid to their corresponding coenzyme A

(CoA) esters, 4-coumaroyl-CoA and cinnamoyl-CoA, is initiated by 4-coumarate/cinnamate:CoA ligase (4CL). These CoA ester compounds are subsequently condensed with three malonyl-CoA via the sequential action of chalcone synthase (CHS) and chalcone isomerase (CHI) to form flavanones. These flavanones are modified further into flavones via the action of flavone synthase (FNS) [10]. Recently, we demonstrated that the codon optimization of flavonoid biosynthetic genes originated from plants, and the heterologous expression of these genes in a recombinant *Streptomyces venezuelae* DHS2001 [2] supplemented with phenylpropanoic acid substrates, were shown to result in the generation of a diverse range of flavonoids, including flavanones and flavones [7,8]. In this study, we present a metabolic engineering strategy to enhance the flavonoid productivity in a recombinant *S. venezuelae* by increasing the availability of malonyl-CoA, the common precursor in flavonoid biosynthesis [10]. With the introduction of malonyl-CoA synthetase MatB and the putative dicarboxylate carrier protein MatC from *Streptomyces coelicolor*, the recombinant production of flavonoids in *S. venezuelae* [7,8] was substantially increased, reaching up to 40 mg/l of flavanones and 30 mg/l of flavones.

The DNA fragments *matB* and *matC* were isolated by PCR from the genomic DNA of *S. coelicolor* using the following primer sets: *matB* forward, 5'-CGAGAATTCCTCTCGGGCAACAGCTAA-3' (*Bam*HI site underlined), *matB* reverse, 5'-CGTCTGTCAGTGACCAGGGCGAGGA ACT-3' (*Pst*I site underlined); and *matC* forward, 5'-TCCTCTAGACAGTCAGGTCAGCACTCG-3' (*Xba*I site underlined), *matC* reverse, 5'-CTGGAATTCCTGTCAGGGATCCGCGAGACGGGGC-3' (*Bam*HI-*Pst*I sites underlined). Each PCR product of *matB* and *matC* was cloned into pLitmus 28 (New England Biolabs) and sequenced to confirm their identities. The *Bam*HI-*Pst*I digested DNA fragment of *matB* was placed into the same sites of the plasmid pLitmus 28 containing *matC*. The resulting plasmid containing

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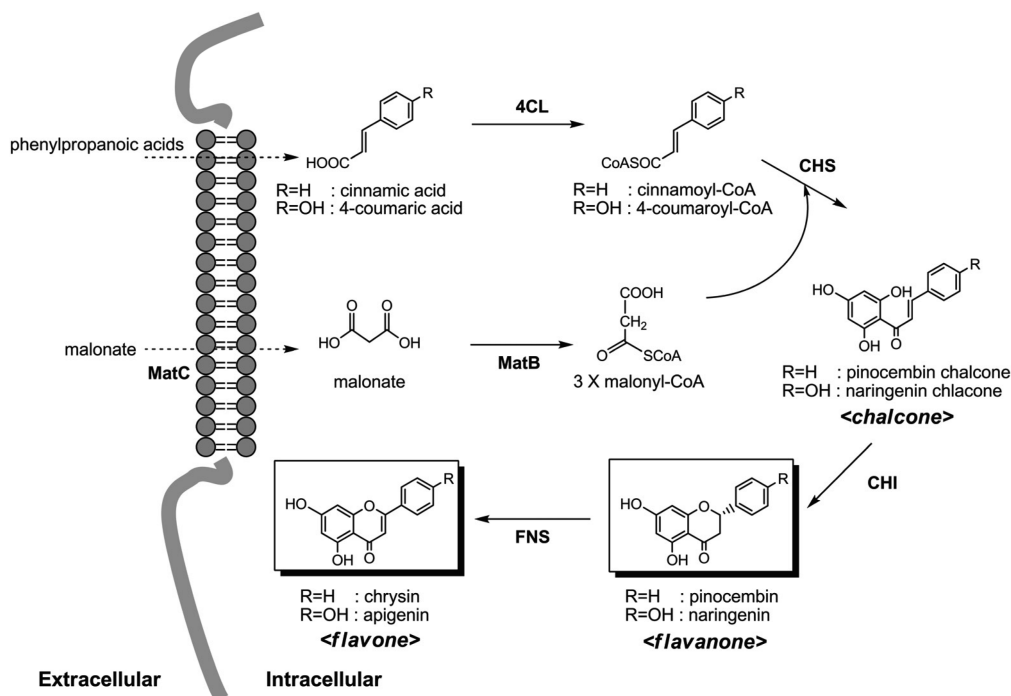


Fig. 1. Engineered pathways for increased flavonoid biosynthesis in *Streptomyces venezuelae*.

Exogenous malonate and phenylpropanoic acids including 4-coumaric acid and cinnamic acid were supplemented into the culture broth of the recombinant *S. venezuelae* strains. Enzyme abbreviations: 4CL, cinnamate/coumarate:CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase; FNS, flavones synthase; MatB, malonyl-CoA synthetase; MatC, dicarboxylate carrier protein.

matB-matC was digested with *XbaI-SpeI* and inserted into the *SpeI* site of the derivative of pSET152 [3] in which the apramycin resistance gene [*aac(3)IV*] was replaced by the kanamycin resistance gene (*aphII*). The plasmid pSET152 containing *matB-matC* was integrated into chromosomal DNA of *S. venezuelae* mutant DHS2001 [2], designated as YJ309. For flavanone biosynthesis, the *chi* DNA fragment from *Arabidopsis thaliana* (*atCHI*) (GenBank Accession No. NM_115370) with the *XbaI* site upstream of the ribosome binding site AGGAGG and the *SpeI-HindIII* site downstream of the stop codon were designed to optimize their codon usage for *S. venezuelae* [8] and synthesized by Genotech, Inc. (Daejeon, Korea). The plasmid harboring *4cl* from *S. coelicolor* (*ScCCL*), a codon-optimized *chs* from *Arabidopsis thaliana* (*atCHSop*), and a codon-optimized *atCHI* (*atCHIop*) were constructed as previously described [6] and introduced into the engineered *S. venezuelae* DHS2001 and YJ309, thereby generating YJ249 and YJ320, respectively. For flavone biosynthesis, an expression plasmid was constructed via the addition of *ScCCL-atCHSop-atCHIop* to the pYJ858 plasmid [7] expressing a codon-optimized flavone synthase gene from *Petroselinum crispum* (*pcFNSop*). The resulting plasmid was also introduced into *S. venezuelae* DHS2001 and YJ309, thus generating YJ273 and YJ341, respectively. For the production of heterologous flavonoids, the recombinant strains were cultivated for 66 h at 30°C in 5 ml of R2YE

liquid medium [4] supplemented with 1.2 mM 4-coumaric acid or cinnamic acid as previously described [7,8]. In order to improve flavonoid production, 2.0 g/l of sodium malonate was added. The cultures of *S. venezuelae* mutant strains were extracted with 1 volume of ethyl acetate and analyzed via HPLC–electrospray ionization–mass spectrometry (HPLC–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 positive-ion mode, as described in the previous study [7, 8]. Quantification was conducted by selecting mass pairs specific for the selected analytes to detect the following transitions of the parent ions to the product ions in multiple reactions monitoring (MRM) mode in MS/MS: naringenin 273>153; pinocembrin 257>153; apigenin 271>153; and chrysin 255>153. Naringenin, pinocembrin, apigenin, and chrysin were employed as authentic standards. Authentic standards and sodium malonate were purchased from Sigma-Aldrich. Three separate cultivations and independent extractions were carried out.

HPLC–ESI–MS/MS analysis results demonstrated that approximately 5.2 mg/l of naringenin and 7.1 mg/l of pinocembrin were generated by *S. venezuelae* YJ249 supplemented with 1.2 mM 4-coumaric acid and cinnamic acid, respectively (lower panels of Fig. 2A and 2B, and 2E). A recent study described the improved production of heterologous flavonoids via the grafting of the malonate

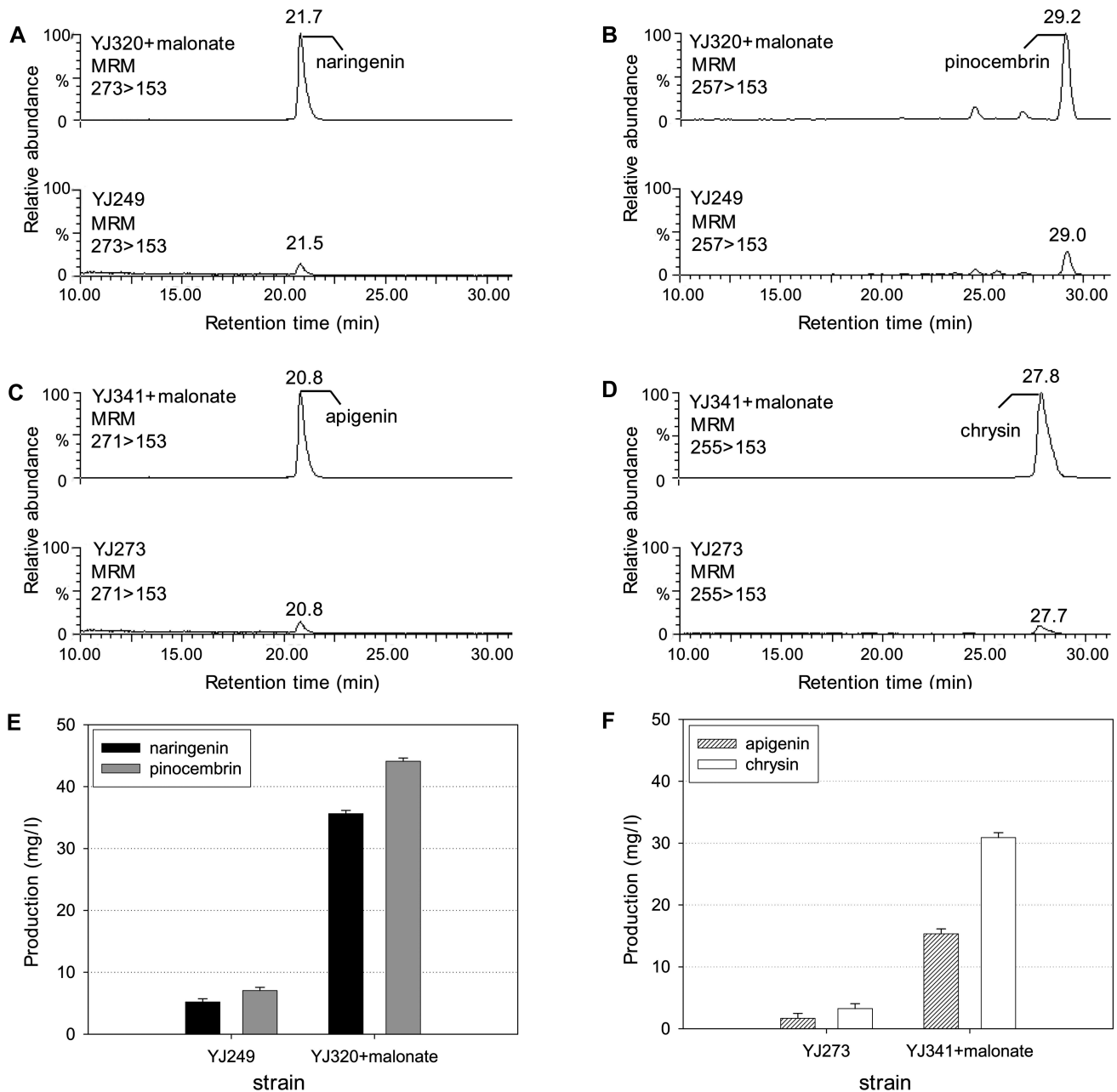


Fig. 2. HPLC–ESI–MS/MS analysis of flavonoids obtained from the recombinant *S. venezuelae* strains. Chromatograms of culture extracts obtained from *S. venezuelae* YJ249 and YJ320 supplemented with (A and B) 4-coumaric acid and cinnamic acid, respectively (lower panels), plus malonate (upper panels); and YJ273 and YJ341 supplemented with (C and D) 4-coumaric acid and cinnamic acid, respectively (lower panels), plus malonate (upper panels). The bar graph indicates the production of (E) flavanones and (F) flavones obtained from the engineered *S. venezuelae* mutant strains.

assimilation operon *Rhizobium trifoli matB* and *matC* into recombinant *E. coli* [5]. In order to increase flavonoid production in *S. venezuelae*, we coexpressed *S. coelicolor matB* and *matC* together with flavonoid biosynthetic genes in the mutant strain of *S. venezuelae*, and supplemented with 2.0 g/l sodium malonate. Combination of the expression of the *matB-matC* (strain YJ320) and supplementation of

exogenous malonate in the recombinant strain led to approximately 7-fold (35.6 mg/l) and 6-fold (44.1 mg/l) enhanced productions of naringenin and pinocembrin, respectively, compared with the levels produced by YJ249 (upper panels of Fig. 2A and 2B, and 2E).

Similarly, flavone production was increased by the introduction of the foreign malonate assimilation pathway

and malonate supplementation. The initial production of flavones, apigenin and chrysin, produced by *S. venezuelae* mutant expressing flavone biosynthetic genes (strain YJ273) was approximately 1.7 mg/l and 3.3 mg/l, respectively, as shown in the lower panels of Fig. 2B and 2D, and 2F. However, approximately 15.3 mg/l of apigenin and 30.9 mg/l of chrysin were generated from the *matB/matC*-integrated mutant YJ341 by feeding malonate (upper panels of Fig. 2C and 2D, and 2F), evidencing a 9-fold enhanced production of apigenin and chrysin compared with that generated by YJ273.

In this study, we successfully accomplished metabolic engineering directed at effective malonate utilization using the *S. coelicolor* MatB-MatC pathway, which resulted in a significant increase in flavonoid production in *S. venezuelae*. To the best of our knowledge, this is the first report regarding the application of a *S. coelicolor*-derived malonate assimilation operon for the improvement of flavonoid production. *R. trifolii matB-matC* has been frequently used to generate more malonyl-CoA for polyketide biosynthesis, including flavonoid biosynthesis [5, 6]. A recent report has elucidated the molecular details of the previously uncharacterized *S. coelicolor* MatB and employed it in the synthesis of diverse CoA-linked extender units [1]. Thus, we may anticipate that the positive role of MatB-MatC from *S. coelicolor* toward flavonoid biosynthesis and the promiscuity of MatB toward malonate substrate can be harnessed for enhanced polyketide production and the generation of a variety of unnatural products, respectively.

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