Article

Heterologous Biosynthesis of Prenylflavonoids in *Escherichia coli* Based on Fungus Screening of Prenyltransferases

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ABSTRACT: Flavonoids are natural products with high biological activity and potential applications. Prenylation increases the lipophilicity of flavonoids, endowing them with specific functions, selectivity, and pharmacological properties. However, traditional methods of plant extraction and chemical synthesis are insufficient to meet the demand for prenylflavonoids. Heterologous biosynthesis of prenylflavonoids in microorganisms provides an alternative approach. Compared with plant prenyltransferases, microbial prenyltransferases showed broad substrate specificity, which is more conducive to the biosynthesis of diverse prenylflavonoids. In this study, we cloned 31 dimethylallyltryptophan synthase prenyltransferases from five fungal species and tested candidate substrates. The products of Ad03 and Ao01 were identified, resulting in two unnatural prenylflavonoids and four natural prenylflavonoids. We constructed the isopentenol utilization pathway in *Escherichia coli* to develop the efficient dimethylallyl diphosphate synthesis pathway for 6-prenylsilybin (6-PS) synthesis. By optimizing the whole cell catalysis and two-phase reaction system, the 6-PS production titer reached 176 mg/L and the yield of silybin was 88%. Our study provides an efficient method for prenylflavonoids production.
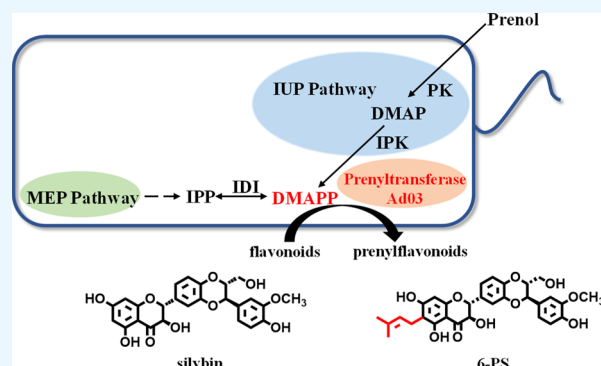
1. INTRODUCTION

Flavonoids are widely distributed in plant secondary metabolites with various biological and pharmacological activities. Prenylation enhances the lipophilicity of flavonoids, improves their affinity to cell membranes, and enhances their interaction with target proteins.[1−6] Compared to flavonoids, prenylflavonoids have many advantages in terms of bioavailability and bioactivity, such as cytotoxicity, antibacterial activity, and antioxidant activity.[7] For example, 8-prenylapigenin exerted a pronounced toxicity in H4IIE cells, while the nonprenylated apigenin showed no cytotoxic activity.[8]

Prenyltransferases (PTs) are responsible for the prenylation of flavonoids in nature. Plant flavonoid PTs are membrane-bound proteins belonging to the UbiA superfamily. They usually have strict substrate and donor specificity, and heterologous expression is difficult. Although prenylflavonoids such as 8-prenylnaringenin[9] and icaritin[10] have been produced by engineered microorganisms with plant PTs, their production is very low. Unlike plant PTs, most microbial PTs are soluble proteins from the dimethylallyltryptophan synthase (DMATS) superfamily. Microbial PTs may not participate in the synthesis of prenylflavonoids naturally, but because of their broad substrate spectrum, they can also catalyze the prenylations of several flavonoids with the dimethylallyl diphosphate (DMAPP) donor.[11−16] As soluble

proteins, they are suitable for heterologous expression in *Escherichia coli*.[17] In addition, due to the broad substrate spectrum of microbial PTs, they could be employed for the synthesis of structurally diverse prenylflavonoids, including some unnatural prenylflavonoids.

Prenylflavonoids are often rare in nature, making their identification and availability for practical applications challenging.[18] Chemical synthesis is difficult to satisfy the demands for high stereochemistry and is not friendly to the environment.[17,19] Therefore, we speculated that the efficient heterologous expression of PTs in microorganisms combined with whole-cell catalysis is an effective method to produce prenylflavonoids.

However, the prenylflavonoid production often suffered from an inefficient DMAPP/IPP supply in the *E. coli* chassis. To increase the supply of DMAPP, the biosynthetic pathways of MVA and MEP were enhanced.[20−22] For example, the truncated form of 3-methylglutaryl coenzyme A reductase

Table 1. List of Plasmids and Strains Used in This Study

	description	source
	Plasmids	
pGEX-4T-1	E. coli expression vector	novagen
pGEX-4T-1-Ad01	pGEX-4T-1 plasmid containing the gene encoding Ad01	in this study
pGEX-4T-1-Ad02	pGEX-4T-1 plasmid containing the gene encoding Ad02	in this study
pGEX-4T-1-Ad03	pGEX-4T-1 plasmid containing the gene encoding Ad03	in this study
pGEX-4T-1-Ad04	pGEX-4T-1 plasmid containing the gene encoding Ad04	in this study
pGEX-4T-1-Ad05	pGEX-4T-1 plasmid containing the gene encoding Ad05	in this study
pGEX-4T-1-Ao01	pGEX-4T-1 plasmid containing the gene encoding Ao01	in this study
pGEX-4T-1-Ao02	pGEX-4T-1 plasmid containing the gene encoding Ao02	in this study
pGEX-4T-1-Ao03	pGEX-4T-1 plasmid containing the gene encoding Ao03	in this study
pGEX-4T-1-Ao04	pGEX-4T-1 plasmid containing the gene encoding Ao04	in this study
pGEX-4T-1-Ao05	pGEX-4T-1 plasmid containing the gene encoding Ao05	in this study
pGEX-4T-1-Ao06	pGEX-4T-1 plasmid containing the gene encoding Ao06	in this study
pGEX-4T-1-Ao07	pGEX-4T-1 plasmid containing the gene encoding Ao07	in this study
pGEX-4T-1-Ao08	pGEX-4T-1 plasmid containing the gene encoding Ao08	in this study
pGEX-4T-1-Ao09	pGEX-4T-1 plasmid containing the gene encoding Ao09	in this study
pGEX-4T-1-Ag01	pGEX-4T-1 plasmid containing the gene encoding Ag01	in this study
pGEX-4T-1-Ag02	pGEX-4T-1 plasmid containing the gene encoding Ag02	in this study
pET28a-Cp01	pET28a plasmid containing the gene encoding Cp01	in this study
pET28a-Cp02	pET28a plasmid containing the gene encoding Cp02	in this study
pET28a-Cp03	pET28a plasmid containing the gene encoding Cp03	in this study
pET28a-Cp04	pET28a plasmid containing the gene encoding Cp04	in this study
pET28a-Cp05	pET28a plasmid containing the gene encoding Cp05	in this study
pET28a-At01	pET28a plasmid containing the gene encoding At01	in this study
pET28a-At02	pET28a plasmid containing the gene encoding At02	in this study
pET28a-At03	pET28a plasmid containing the gene encoding At03	in this study
pET28a-At04	pET28a plasmid containing the gene encoding At04	in this study
pET28a-At05	pET28a plasmid containing the gene encoding At05	in this study
pET28a-At06	pET28a plasmid containing the gene encoding At06	in this study
pET28a-At07	pET28a plasmid containing the gene encoding At07	in this study
pET28a-At08	pET28a plasmid containing the gene encoding At08	in this study
pET28a-At09	pET28a plasmid containing the gene encoding At09	in this study
pET28a-At10	pET28a plasmid containing the gene encoding At10	in this study
pETDuet-1	E. coli expression vector	Novagen
pCDFDuet-1	E. coli expression vector	Novagen
pETDuet-1-Thim	pETDuet-1 plasmid containing the gene encoding Thim	in this study
pETDuet-1-TTa	pETDuet-1 plasmid containing the gene encoding Thim and TaIPK	in this study
pCDFDuet-1-Ad03	pCDFDuet-1 plasmid containing the gene encoding Ad03	in this study
pCDFDuet-1-Ao01	pCDFDuet-1 plasmid containing the gene encoding Ao01	in this study
	Strains	
BL21	general expression host	Invitrogen
BL21-pGEX	BL21 harboring plasmid pGEX-4T-1	in this study
BL21-Ad01	BL21 harboring plasmid pGEX-4T-1-Ad01	in this study
BL21-Ad02	BL21 harboring plasmid pGEX-4T-1-Ad02	in this study
BL21-Ad03	BL21 harboring plasmid pGEX-4T-1-Ad03	in this study
BL21-Ad04	BL21 harboring plasmid pGEX-4T-1-Ad04	in this study
BL21-Ad05	BL21 harboring plasmid pGEX-4T-1-Ad05	in this study
BL21-Ao01	BL21 harboring plasmid pGEX-4T-1-Ao01	in this study
BL21-Ao02	BL21 harboring plasmid pGEX-4T-1-Ao02	in this study
BL21-Ao03	BL21 harboring plasmid pGEX-4T-1-Ao03	in this study
BL21-Ao04	BL21 harboring plasmid pGEX-4T-1-Ao04	in this study
BL21-Ao05	BL21 harboring plasmid pGEX-4T-1-Ao05	in this study
BL21-Ao06	BL21 harboring plasmid pGEX-4T-1-Ao06	in this study
BL21-Ao07	BL21 harboring plasmid pGEX-4T-1-Ao07	in this study
BL21-Ao08	BL21 harboring plasmid pGEX-4T-1-Ao08	in this study
BL21-Ao09	BL21 harboring plasmid pGEX-4T-1-Ao09	in this study
BL21-Ag01	BL21 harboring plasmid pGEX-4T-1-Ag01	in this study
BL21-Ag02	BL21 harboring plasmid pGEX-4T-1-Ag02	in this study
BL21-Cp01	BL21 harboring plasmid pET28a-Cp01	in this study
BL21-Cp02	BL21 harboring plasmid pET28a-Cp02	in this study
BL21-Cp03	BL21 harboring plasmid pET28a-Cp03	in this study
BL21-Cp04	BL21 harboring plasmid pET28a-Cp04	in this study
BL21-Cp05	BL21 harboring plasmid pET28a-Cp05	in this study
BL21-At01	BL21 harboring plasmid pET28a-At01	in this study
BL21-At02	BL21 harboring plasmid pET28a-At02	in this study
BL21-At03	BL21 harboring plasmid pET28a-At03	in this study
BL21-At04	BL21 harboring plasmid pET28a-At04	in this study
BL21-At05	BL21 harboring plasmid pET28a-At05	in this study
BL21-At06	BL21 harboring plasmid pET28a-At06	in this study
BL21-At07	BL21 harboring plasmid pET28a-At07	in this study
BL21-At08	BL21 harboring plasmid pET28a-At08	in this study
BL21-At09	BL21 harboring plasmid pET28a-At09	in this study
BL21-At10	BL21 harboring plasmid pET28a-At10	in this study
TTa	BL21 harboring plasmid pETDuet-1-Thim-TaIPK	in this study
TTa-Ad03	BL21 harboring plasmid pETDuet-1-Thim-TaIPK and pCDFDuet-1-Ad03	in this study
TTa-Ao01	BL21 harboring plasmid pETDuet-1-Thim-TaIPK and pCDFDuet-1-Ao01	in this study

(tHMG1) was used in the MVA pathway.[23] In recent years, an artificial pathway has been established for the DMAPP/IPP synthesis. The isopentenol utilization pathway (IUP) produces DMAPP from prenol through consecutive two-step reactions catalyzed by promiscuous kinase (PK) and isopentenyl phosphate kinase (IPK).[24,25] The production titer of 6-prenylnaringenin was increased from 2.6 mg/L (MVA + MEP) to 7.4 mg/L (IUP + MEP) by enhancing the donor DMAPP synthesis pathway and further increased to 69.9 mg/L by optimizing the process for whole-cell catalysis.[26] Through coupling the IUP pathway, the production titer of licoflavanone was increased to 142.1 mg/L in a shake flask.[27]

Flavonoids such as silybin (sil), daidzein (dai), and baicalein (bai) are beneficial to human health and widely used. The prenylation of these flavonoids may give compounds with improved therapeutic efficacy. Therefore, this research is committed to pioneering the synthesis of structurally diverse prenylflavonoids. We provide a microbial whole-cell catalysis platform to synthesize prenylflavonoids efficiently.

2. MATERIALS AND METHODS

2.1. Materials, Chemicals, and Media.
Flavonoids such as silybin, baicalein, and daidzein were purchased from Nantong Feiyu Biological Technology Co., Ltd. (Nantong, China). Prenol (3-methyl-2-buten-1-ol) was purchased from Sinopharm Chemical Reagent (Shanghai, China). DMAPP was purchased from Sigma Chemical Co. (St. Louis, MO). LB medium contained 10 g/L tryptone, 5 g/L yeast extract, and 10 g/L sodium chloride. TB-glucose, TB-glycerol (TB-Gly), TB-maltose, TB-lactose (TB-Lac), and TB-arabinose contained the same components (12 g/L tryptone, 24 g/L yeast extract, 2.32 g/L KH_2PO_4, and 12.54 g/L K_2HPO_4) but a different carbon source (10 g/L glucose, 10 g/L glycerol, 10 g/L maltose, 10 g/L lactose, and 10 g/L arabinose, respectively).

2.2. Bioinformatics Analysis.
A public Hidden Markov Model (PF11991, http://pfam.xfam.org/) was used to predict the PTs of *Aspergillus nidulatus*, *Aspergillus oryzae*, *Claviceps purpurea*, *Aspergillus terreus*, and *Aspergillus niger*. The amino acid sequences of the candidate PTs were analyzed using MAFFT software, and the phylogenetic tree was constructed using the neighbor-joining method with Geneious R8 software, with 1000 bootstrap replicates. Accession numbers of the related PTs are listed in Table S3.

2.3. Plasmid Construction.
All plasmids and strains used in this study are listed in Table 1, and the relevant primers are listed in Table S1. The strains were grown at 37 °C in TB-Gly medium supplemented with antibiotics when required. PT candidate genes were deposited in the Registry and database of bioparts for synthetic biology (https://www.biosino.org); accession numbers are OENC366060-OENC366090. They were constructed on pGEX-4T-1 plasmids digested by *XhoI* and *BamHI* or pET-28a plasmids digested by *NcoI* and *SalI* to form BL21-PTs strains. The IUP was reconstructed in *E. coli* by introducing PK and IPK. The PK gene from *E. coli* (*Thim*) and the codon-optimized IPK gene from *Thermoplasma acidophilum* (*TaIPK*) were selected. *Thim* was constructed on the pETDuet-1 plasmid digested by *NcoI* and *BamHI* to form pETDuet-1-Thim strain. Then, *TaIPK* was constructed onto the plasmid pETDuet-1-Thim cut by *NdeI* and *XhoI*, respectively, to form the IUP strain pETDuet-1-Thim-TaIPK (TTa).

2.4. Functional Identification of Candidate PTs.
The BL21-PTs strains and negative control (BL21) were cultured

overnight in 5 mL of LB medium supplemented with the appropriate antibiotics. Seed culture (1 mL) was added to 50 mL of fresh LB medium containing the appropriate antibiotics. The cultures were induced to express candidate PTs by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.2 mM at an OD_{600} of ~0.8, followed by incubation at 16 °C for 16 h. The cells were harvested by centrifugation at 8000g for 5 min and suspended in 3 mL of 50 mM Tris−HCl buffer (pH 7.5) and disrupted using a French press (25 kpsi). Cell debris was removed by centrifugation at 12,000g for 10 min, and the supernatant was used as a crude enzyme solution for enzymatic assays.

Enzymatic assays for PTs were carried out in a 50 μL volume containing 50 mM Tris−HCl buffer (pH 7.5), 40 μL of crude enzyme solution, 0.4 mM DMAPP as the prenyl donor, and 0.4 mM flavonoid substrates as the prenyl receptor for 10 h at 37 °C. The reaction products were extracted by adding 100 μL of ethyl acetate, and the organic phase was evaporated and dissolved in 50 μL of methanol for high-performance liquid chromatography (HPLC).

2.5. DMAPP Standard and Sample Preparation.
The DMAPP standard was obtained by dissolving 100 ng of DMAPP in 1 mL of 10 mM Tris−HCl buffer (containing 1 mM EDTA, pH 8.0): methanol = 1:9.

The TTa sample was obtained as follows. The TTa strain was cultured in 5 mL of TB-Gly medium supplemented with the appropriate antibiotics. Seed culture (250 μL) was added to 25 mL of fresh TB-Gly medium containing the appropriate antibiotics. The cultures were induced to express recombinant Thim and TaIPK by adding 0.2 mM IPTG and 20 mM prenol at an OD_{600} of ~0.8, followed by incubation at 30 °C for 6 h. The cells were harvested by centrifugation at 8000g for 10 min and washed with 200 μL of 10 mM Tris−HCl buffer (containing 1 mM EDTA, pH 8.0) for 3 times. Then, the cells were suspended with 400 μL of lysis buffer [10 mM Tris−HCl buffer (containing 1 mM EDTA, pH 8.0): methanol = 1:9] and placed overnight at −20 °C. The cells were vortexed and centrifuged at 4 °C and 15,000 g for 10 min. The supernatant was a mass spectrometry sample. The BL21 sample was obtained in the same way. All metabolite concentrations are reported as means ± SD of three biological replicates.

2.6. Construction of Strains for Whole-Cell Catalysis.
*Ad*03 from *Aspergillus nidulans* and *Ao*01 from *A. oryzae* were constructed onto the pCDFDuet-1 plasmid cut by *NcoI* and *BamHI*, respectively, to form PT plasmids: pCDFDuet-1-Ad03 and pCDFDuet-1-Ao01. The plasmids of PT and TTa were simultaneously transferred into BL21 (DE3) to construct strains containing the IUP.

The recombinant strain TTa-Ad03 was inoculated into 5 mL of fresh TB-Gly medium containing 100 μg/mL ampicillin and 50 μg/mL spectinomycin. The recombinant strains were grown at 37 °C until the OD_{600} reached 0.8. 0.2 mM IPTG was added and induced at 30 °C for 6 h. Then, silybin (20 mg/L) and prenol (20 mM) were added to the broth. The fermentation broths were incubated at 30 °C and 250 rpm for 24 h. 200 μL of ethyl acetate was added to 200 μL of the fermentation broth for shaking extraction for 1 h. The supernatant was harvested by centrifugation at 12,000g for 1 min. The extract was evaporated and resuspended in 50 μL of methanol for HPLC analysis.

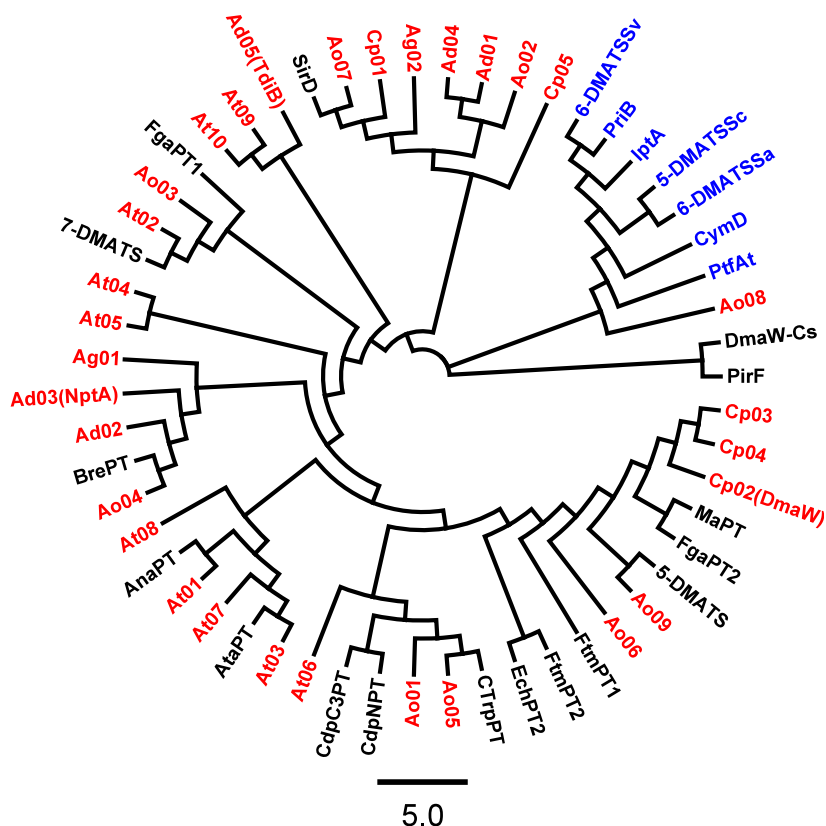The optimized fermentation method was to inoculate the recombinant strain TTa-Ad03 into fresh TB-Lac medium

Figure 1. Phylogenetic analysis of DMATS PTs. The tree was constructed via the neighbor-joining algorithm. Accession numbers are provided in Table S3. Reported bacterial source PTs are shown in blue font, reported fungal source PTs are shown in black font, and the fungal source PTs mined for this study are shown in red font.

containing the corresponding antibiotics. The recombinant strain was cultured at 37 °C to OD_{600} of 0.8. 0.2 mM IPTG (0.2 mM) was added and induced at 30 °C for 6 h. The cells were harvested by centrifugation at 4000g for 10 min and suspended with 50 mM Tris−HCl buffer (pH 8.0) until OD_{600} was 200. The whole-cell catalysis reaction system consisted of 200 μL of cell suspension at an OD_{600} of 200, 200 mg/L silybin, and 3 mM prenol. The fermentation solution was cultured at 30 °C and 250 rpm for 24 h. The samples were extracted with twice the volume of ethyl acetate, dissolved in 50 μL methanol, and analyzed by HPLC. All product concentrations are reported as means ± SD of three biological replicates.

2.7. Optimization of the Process for Whole-Cell Catalysis. The whole-cell catalysis process of silybin using the TTa-Ad03 strain was optimized. The TTa-Ad03 strain was inoculated in TB-Gly medium containing 100 μg/mL ampicillin and 50 μg/mL streptomycin and grown at 37 °C until OD_{600} reached 0.8. A final concentration of 0.2 mM IPTG was added, followed by incubation at 30 °C for 6 h. Then, silybin and prenol were added. The fermentation solution was incubated at 30 °C and 200 rpm for 24 h. The effects of different media (LB, TB-glucose, TB-Gly, TB-maltose, TB-Lac, or TB-arabinose), silybin (10, 20, 50, 100, 200, 500, or 1000 mg/L), prenol (0, 1, 3, 5, 7, 10, 15, 20, or 30 mM), and cell density OD_{600} (20, 50, 100, 200 or 500) were determined. The samples were extracted with twice the volume of ethyl acetate, dissolved in 50 μL of methanol, and analyzed by HPLC. All product concentrations are reported as means ± SD of three biological replicates.

2.8. HPLC and UPLC−MS/MS Spectrometry Analysis. HPLC analysis was performed using a Shimadzu LC 20A system (Shimadzu, Kyoto, Japan) equipped with a LC20ADXR pump, an autosampler, a binary pump, and a diode array detector. To analyze the products, chromatographic separation was performed at 35 °C with a Welch Boltimate C18 (2.7 μm, 2.1 × 100 mm) column. The gradient elution system consisted of 0.1% acetic acid/water (A) and acetonitrile (B). The extract was separated using the following gradient: 0−2 min (15% B), 2−16 min (15−70% B), 16−18 min (95% B), and 18−20 min (15% B). The flow rate was kept at 0.45 mL/min. The products were monitored at a 290 nm absorbance.

Mass spectrometry was performed on a UPLC instrument combined with a QTRAP 6500+ MS system equipped with an electrospray ionization (ESI) source (AB SCIEX). Instrument control and data acquisition were performed using Analyst 1.6.3 software (AB SCIEX), and data processing was performed using MultiQuant 3.0.2 software (AB SCIEX). The compounds were quantified by calculating the area of each individual peak and comparing it to standard curves. DMAPP was separated with an XSelect HSS T3 column (2.5 μm, 3.0 mm × 100 mm). The UPLC method for DMAPP is shown in Table S4. The optimized ESI operating parameters for negative mode were as follows: IS, −4500 V; CUR, 35 psi; transmission electron microscopy 500 °C; GS1, 50 psi; GS2, 50 psi. All analytes were detected using the multiple reaction monitoring (MRM) mode, and the specific MRM parameters for each analyte are given in Table S5.

2.9. Nuclear magnetic resonance Analysis. Nuclear magnetic resonance (NMR) spectra were generated using the
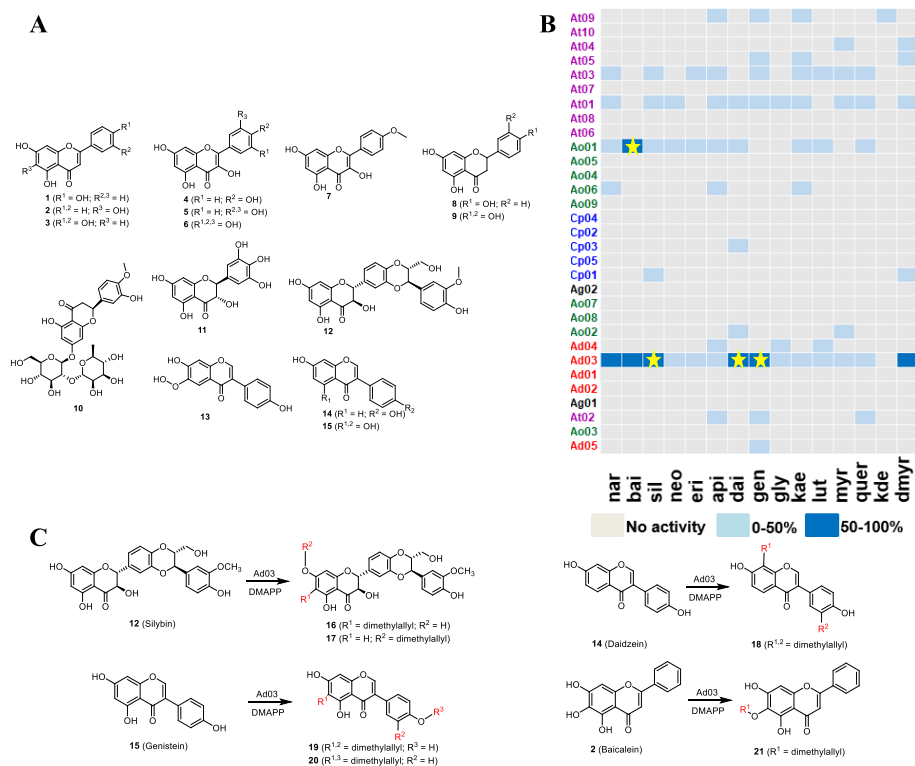
Figure 2. Functional characterization of candidate PTs. (A) Structures of the flavonoids assayed as prenyl acceptors. (B) HPLC analysis of different flavonoid substrates catalyzed by PT candidate genes. Gray is inactive, light blue is less than 50% conversion, and dark blue is greater than 50% conversion. The yellow stars represent the product structures are determined. (C) Identified products from enzymatic reactions by Ad03 with silybin (**12**), daidzein (**14**), and genistein (**15**) and product from enzymatic reactions by Ao01 with baicalein (**2**).

Bruker ADVANCE III 500 instrument (^{1}H: 500 MHz, ^{13}C: 125 MHz; Bruker, Billerica, MA, USA), with chemical shift values expressed as ppm relative to tetramethylsilane (δ_H 0.00 and δ_C 0.00 ppm), using residual chloroform (δ_H 7.26 and δ_C 77.16 ppm) as a standard.

3. RESULTS AND DISCUSSION

3.1. Mining and Identification of PTs in the DMATS Superfamily.
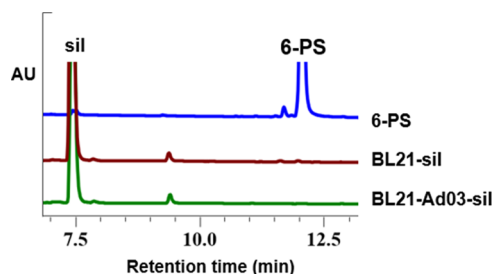The transcriptome data of *A. nidulatus*, *A.*

Figure 3. Production of 6-PS in the BL21 strain was analyzed by HPLC. HPLC analysis of 6-PS synthesis of Ad03 in wild-type chassis BL21.

oryzae, *C. purpurea*, *A. terreus*, and *A. niger* were obtained from a public database. A specific Hidden Markov Model (PF11991) was used to predict the PTs. We predicted five candidate genes from *A. nidulans*, nine candidate genes from *A. oryzae*, five candidate genes from *C. purpurea*, ten candidate genes from *A. terreus*, and two candidate genes from *A. niger*. These candidate genes were cloned using cDNA as a template

and overexpressed in *E. coli*. To further analyze the relationships between the candidate PTs and other known DMATS PTs, we selected the DMATS PTs mined in this study as the candidate PTs, and we selected functionally identified DMATS PTs from the published literature as known DMATS PTs to generate a phylogenetic tree (Figure 1). We found that the candidate PTs have a closer evolutionary relationship with the fungal source PTs. Ad03, Ad05 and Cp02 are reported PTs NptA, TdiB and DmaW, while other candidate PTs are novel. Ad03 (NptA) can catalyze the prenylation of tryptophan to participate in nidulanin A synthesis in *Aspergillus nidulans*.[28] Ad05 (TdiB) can catalyze the prenylation of didemethylasterriquinone D to produce asterriquinone C-1, a step in the biosynthesis of aterrequinone A.[29] Cp02 (DmaW) can catalyze the prenylation of tryptophan and its derivatives at C-4.[30]

However, the catalytic activity of all candidate PTs with respect to flavonoids is unknown. We selected 15 flavonoid substrates of different flavonoid subclasses and systematically identified the functions of these 31 candidate PTs. The flavonoid substrates include apigenin (api, **1**), baicalein (bai, **2**), luteolin (lut, **3**), kaempferol (kae, **4**), quercetin (quer, **5**), myricetin (myr, **6**), kaempferide (kde, **7**), naringenin (nar, **8**), eriodictyol (eri, **9**), neohesperidin (neo, **10**), dihydromyricetin (dmyr, **11**), silybin (sil, **12**), glycitein (gly, **13**), daidzein (dai, **14**), and genistein (gen, **15**). The BL21-PTs strains were cultured to induce the expression of candidate PTs, and crude enzyme solution was obtained for the in vitro enzymatic reaction. The products in the reaction system were analyzed by HPLC. Compared with negative control (nonloaded plasmid), any additional peaks might correspond to new products
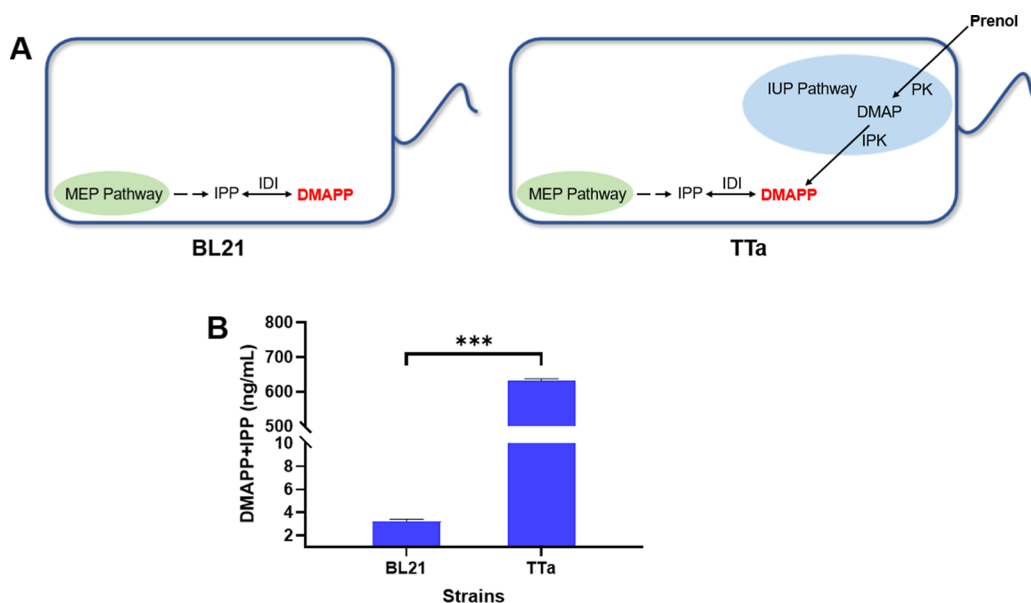
Figure 4. Construction of an *E. coli* chassis-enhanced DMAPP synthesis system. (A) Schematics of different chassis strains. The wild-type strain BL21 contains the native MEP pathway of *E. coli* to synthesize DMAPP; the exogenous IUP pathway was introduced by the TTa strain to enhance the supply of DMAPP. (B) DMAPP + IPP content was quantified by mass spectrometry. DMAPP with 100 ng/mL was used as the standard sample to detect the DMAPP + IPP content in BL21 and TTa strains.
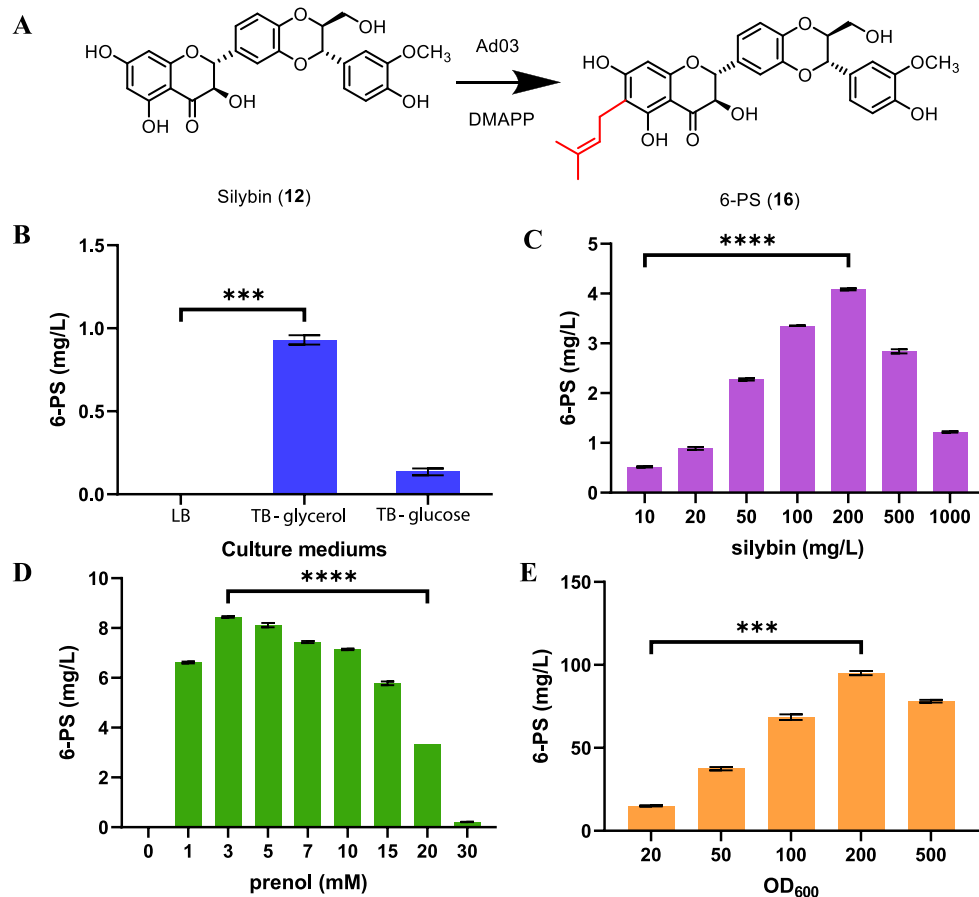

Figure 5. Biosynthesis of 6-PS in the engineered *E. coli* strain. (A) Prenylation of silybin by Ad03. (B) Effect of culture mediums on 6-PS production. (C) Effect of silybin concentration on 6-PS production. (D) Effect of prenol concentration on 6-PS production. (E) Effect of OD_{600} on 6-PS production.

formed, and the samples with high yield were identified by mass spectrometry. HPLC analysis showed that Ad03 could
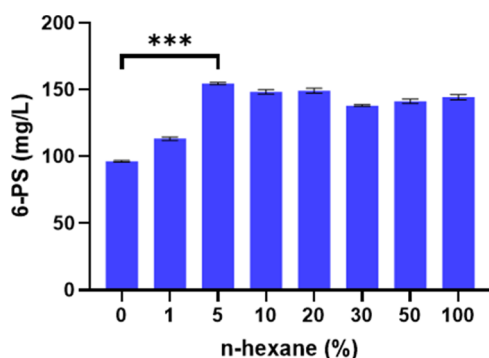
Figure 6. Effect of the organic phase on the production of 6-PS. The effect of 0−100% *n*-hexane on the production of 6-PS.

efficiently catalyze the prenylation of various types of flavonoids, while Ao01 could efficiently catalyze only the prenylation of baicalein.

Compared with the reported literature data,[31] the minor prenylation product of silybin was elucidated as 7-O-prenylsilybin (**17**). The major prenylation product share the same molecular weight with product **17**, while 1H shift of methylene in the prenyl group was lower than that in compound **17** (3.52 ppm VS 4.63 ppm), which suggested that the prenyl group was added on a carbon of the aromatic ring. The HMBC of methylene-H in the prenyl group with C7 and H8 with C9 suggested that the major prenylation product **16**, named as 6-prenylsilybin (6-PS), was prenylated on C6 of silybin. One of diprenylation products of genistein was elucidated as lupalbigenin **19** according to the literature reported data.[32,33] Compared the ^{1}H NMR spectrum of prenylation products **19** and **20**, one of the prenyl group in compound **20** was O-prenylation and the position was confirmed as 4′-O-prenylation according NOE correlation between H1″ (4.54 ppm) and H3′ (H5′) (6.98 ppm). The prenylation products **18** and **21** were confirmed via comparing their spectra with the literature reported data, and all the spectra were consistent.[34,35] In summary, we obtained two unnatural prenylflavonoids (6-PS, **16** and **20**) and four reported prenylflavonoids (7-O-prenylsilybin, **17**; erysubin F, **18**; lupalbigenin, **19**; and 6-O-prenylbaicalein, **21**) (Figures 2 and S9−S34).

3.2. Engineering an Artificial Pathway for Efficient Biosynthesis of Prenyl Donor DMAPP in *E. coli*. To test the ability of the *E. coli* strain for prenylflavonoids production,

PT Ad03 from *A. nidulans*, which was characterized to catalyze the biosynthesis of 6-PS from silybin, was overexpressed in *E. coli* strain BL21. The resulting strain was tested for the biosynthesis of 6-PS via whole-cell catalysis using silybin as a substrate. However, no detectable 6-PS could be detected by HPLC analysis (Figure 3).

The PTs catalyze the prenylation of flavonoids and require DMAPP as a prenyl donor. Although *E. coli* has a native MEP pathway for DMAPP synthesis, we hypothesized that the DMAPP content in wild-type *E. coli* might not be enough to support the prenylation reaction (Figure 4A). Therefore, we consider introducing exogenous pathways to increase the intracellular DMAPP content. In addition to the MEP pathway, the MVA pathway is a well-known pathway for DMAPP/IPP synthesis, which has also been successfully reconstructed in *E. coli*.[21] However, the long length and complex regulation of the MVA and MEP pathways make it a challenging task to improve the metabolic flux to produce DMAPP. We constructed a two-step IUP in *E. coli*, combining PK enzyme (*Thim* from *E. coli*) and IPK enzyme (*TaIPK* from *T. acidophilum*) to obtain the TTa strain. The contents of DMAPP and IPP in strains were tested by mass spectrometry analysis (Figure 4B). Compared with wild-type strain BL21, DMAPP and IPP contents of the strain TTa were significantly increased, with 636 ng/mL, which was 212 times higher than that of BL21. Therefore, the TTa strain was selected to construct subsequent PT strains.

3.3. Efficient Biosynthesis of Prenylflavonoid 6-PS in the Engineered *E. coli* Strain. To test capacity of the strain TTa for prenylflavonoid synthesis, Ad03 was introduced, and the resulting strain was used for 6-PS production (Figure 5A). As detected by HPLC, 0.91 mg/L 6-PS can be obtained from 20 mg/L silybin, representing a conversion ratio of 0.45%. To further increase the whole-cell catalysis efficiency, we next optimized the culture media, silybin concentration, prenol concentration, and OD_{600}. As shown in Figure 5, the 6-PS production titer was 0.91 mg/L in TB-Gly, which was higher than those in TB-glucose and LB, so we chose to conduct subsequent optimization experiments in TB-Gly medium. When the concentration of silybin was higher than 200 mg/L, the yield decreased significantly, while 6-PS production increased when the addition of silybin was no more than 200 mg/L. The highest 6-PS production titer reached 4.1 mg/L, so 200 mg/L silybin was selected for the following experiment. The same results were observed for prenol. The maximal 6-PS
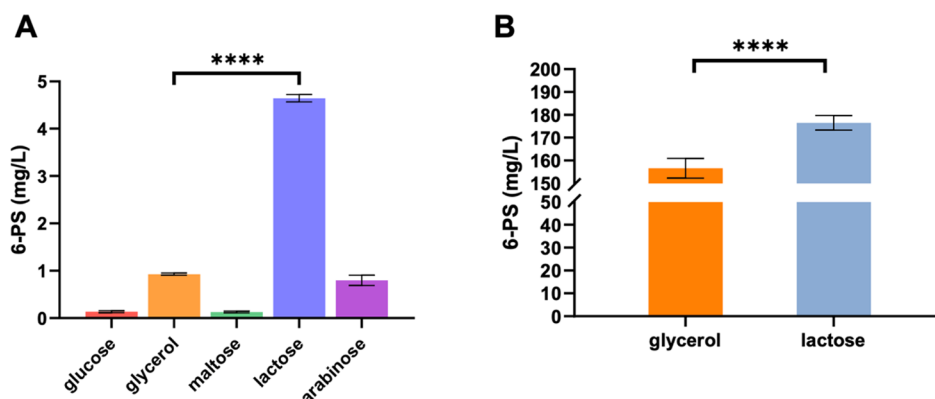

Figure 7. Effect of carbon sources on the production of 6-PS. (A) Effect of carbon sources on 6-PS production. (B) Production of 6-PS with glycerol and lactose as carbon sources in an optimal catalytic system.
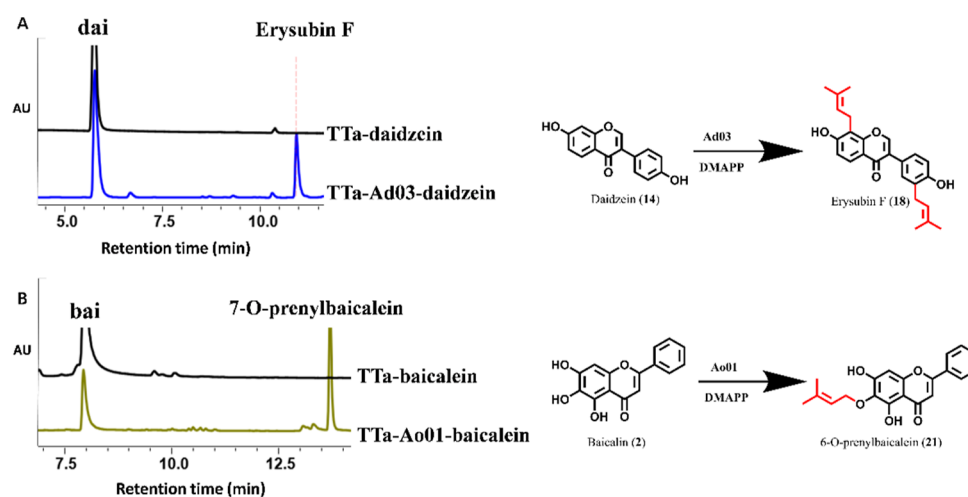
Figure 8. Prenylflavonoid cell factory we constructed can catalyze daidzein and baicalein. (A) HPLC analyses and catalytic reaction of 200 mg/L daidzein catalyzed by Ad03. (B) HPLC analyses and catalytic reaction of 200 mg/L baicalein catalyzed by Ao01.

production titer was 8.4 mg/L when the addition of prenol was 3 mM, so we chose 3 mM prenol to conduct following experiment.

Stabilizing the fermentation solution at a stable cell density (OD_{600}) is also a common strategy for whole-cell catalysis. Choosing a suitable cell density can catalyze the reaction more efficiently. We tested OD_{600} at 20, 50, 100, 200, and 500 with a pH of 7.5 buffer containing 50 mM Tris−HCl to investigate the effect of cell density on the 6-PS production titer . When OD_{600} was from 20 to 200, 6-PS production increased with the increase of cell density, while 6-PS production reduced when OD_{600} was 500. The results showed that the optimal OD_{600} was 200 and the production titer was 95 mg/L (Figure 5E).

3.4. Effects of Organic Reagents and Carbon Sources on 6-PS Production. Two-phase catalysis can change the hydrogen bonding and hydrophobic interactions at the two-phase interface.[36,37] We tested the effect of different organic reagents (*n*-hexane, ethyl acetate, ethyl alcohol, methanol, isopropanol, cyclohexane, and acetonitrile) on the 6-PS production titer and found that *n*-hexane could significantly increase the titer (Figure S35). Further testing of different doses of *n*-hexane showed that 5% of the reaction volume had the best catalytic effect on 6-PS production, with the production titer reaching 154 mg/L and the yield of silybin reaching 77% (Figure 6).

Different carbon sources may affect the metabolism of the TTa strain and the production of 6-PS. Therefore, we tested their effects on 6-PS production. Among 15 carbon sources, 6-PS production was determined in TB-glucose, TB-Gly, TB-maltose, TB-Lac, and TB-arabinose. With lactose as the carbon source, the production titer of 6-PS was greatly increased (Figure 7A). In the optimal catalytic system, the 6-PS titer of lactose as a carbon source reached 176 mg/L, and the yield of silybin was 88%, which was higher than that of glycerol as a carbon source, 154 mg/L (Figure 7B). It is currently the highest output value of prenylflavonoid cell factory.

3.5. Production of Other Prenylflavonoids by the Engineered Platform Strain. So far, we have successfully constructed a DMAPP/IPP high-yielding chassis strain TTa. The target product was successfully detected by introducing PT Ad03. After several rounds of condition optimization and two-phase catalysis, *E. coli* cell factory with a high yield of prenylflavonoid was finally obtained.

But whether this cell factory is universal is a question we want to explore. We first put daidzein, another type of flavonoid substrate, into the constructed TTa-Ad03 strain. The corresponding target product (erysubin F) can be detected at a titer of 53.5 mg/L, indicating that this chassis is suitable for different substrates. Then, we introduced different PTs, such as Ao01, which can produce the corresponding target product (6-O-prenylbaicalein) at a titer of 32 mg/L, indicating that this chassis is suitable for different PTs (Figure 8).

4. CONCLUSIONS

In this study, we cloned 31 DMATS PTs from *A. nidulatus*, *A. oryzae*, *C. purpurea*, *A. terreus*, and *A. niger*. To investigate their catalytic activity, 15 flavonoid substrates were tested. The products of Ad03 and Ao01 were identified, resulting in two unnatural prenylflavonoids and four natural prenylflavonoids. Then, we optimized the synthesis of donor DMAPP in *E. coli* using PK enzyme (*Thim* from *E. coli*) and IPK enzyme (*TaIPK* from *T. acidophilum*) to construct an easily regulated two-step IUP and developed a whole-cell catalysis platform for various natural and unnatural prenylflavonoids production. Through optimizing the whole-cell catalysis, two-phase reaction system, and carbon sources, the production titer of 6-PS increased from 0.91 to 176 mg/L. Collectively, our study provides an effective way for novel prenylflavonoids production and mass production by the development of a microbial whole-cell catalysis platform.

■ ASSOCIATED CONTENT

ⓈI Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.4c05007.

> Primers used in this study, candidate PTs in *A. nidulatus*, *A. oryzae*, *C. purpurea*, *A. terreus,* and *A. niger*, phylogenetic analysis of PTs belonging to the DMATS superfamily, the UPLC method for DMAPP, the specific MRM parameters for DMAPP, HPLC analysis of the production of silybin catalyzed by Ad03, HPLC analysis of the production of genistein catalyzed by Ad03, HPLC analysis of the production of daidzein catalyzed by Ad03, HPLC analysis of the production of baicalein catalyzed by Ao01, LC−MS analyses of the production of silybin

catalyzed by Ad03, LC−MS analyses of the production of genistein catalyzed by Ad03, LC−MS analyses of the production of daidzein catalyzed by Ad03, LC−MS analyses of the production of baicalein catalyzed by Ao01, NMR spectra of compounds 6-PS (**16**), 7-*O*-prenylsilybin (**17**), erysubin F (**18**), lupalbigenin (**19**), **20**, and 6-*O*-prenylbaicalein (**21**), effect of different organic reagents on the production of 6-PS, and NMR data of compounds 6-PS (**16**), 7-*O*-prenylsilybin (**17**), lupalbigenin (**19**), **20**, erysubin F (**18**), and 6-*O*-prenylbaicalein (**21**) (PDF)

Accession Codes

The cloned gene sequences of PTs in this study were submitted in the Registry and database of bioparts for synthetic biology (https://www.biosino.org), accession numbers are as follows: AdPT01 (OENC366060.p), AdPT02 (OENC366061.p), AdPT03 (OENC366062.p), AdPT04 (OENC366063.p), AdPT05 (OENC366064.p), AoPT01 (OENC366065.p), AoPT02 (OENC366066.p), AoPT03 (OENC366067.p), AoPT04 (OENC366068.p), AoPT05 (OENC366069.p), AoPT06 (OENC366070.p), AoPT07 (OENC366071.p), AoPT08 (OENC366072.p), AoPT09 (OENC366073.p), CpPT01 (OENC366074.p), CpPT02 (OENC366075.p), CpPT03 (OENC366076.p), CpPT04 (OENC366077.p), CpPT05 (OENC366078.p), AtPT01 (OENC366079.p), AtPT02 (OENC366080.p), AtPT03 (OENC366081.p), AtPT04 (OENC366082.p), AtPT05 (OENC366083.p), AtPT06 (OENC366084.p), AtPT07 (OENC366085.p), AtPT08 (OENC366086.p), AtPT09 (OENC366087.p), AtPT10 (OENC366088.p), AgPT01 (OENC366089.p), AgPT02 (OENC366090.p).

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Notes

The authors declare no competing financial interest.

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