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TITLE:

Biosynthesis of a flavonol from a flavanone by establishing a one-pot bienzymatic cascade

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KEYWORDS:

flavonol; flavanone; kaempferol; quercetin; biosynthesis; multienzyme; bienzyme; flavanone 3-hydroxylase; flavonol synthase

SUMMARY:

The derivation of a flavonol is crucial for its application in healthcare and the food industry. Here, we provide a detailed protocol for the biosynthesis of a flavonol from a flavanone and discuss the crucial steps and its advantages over other approaches.

ABSTRACT:

Flavonols are a major subclass of flavonoids with a variety of biological and pharmacological activities. Here, we provide a method for the in vitro enzymatic synthesis of a flavonol. In this method, *Atf3h* and *Atfls1*, two key genes in the biosynthetic pathway of the flavonols, are cloned and overexpressed in *Escherichia coli*. The recombinant enzymes are purified via an affinity column and then a bienzymatic cascade is established in a specific synthetic buffer. Two flavonols are synthesized in this system as examples and determined by TLC and HPLC/LC/MS

analyses. The method displays obvious advantages in the derivation of flavonols over other approaches. It is time- and labor-saving and highly cost-effective. The reaction is easy to be accurately controlled and thus scaled up for mass production. The target product can be purified easily due to the simple components in the system. However, this system is usually restricted to the production of a flavonol from a flavanone.

INTRODUCTION:

Flavonols are a major subclass of plant flavonoids and are involved in plant development and pigmentation[1-3]. More importantly, these compounds possess a wide range of health-beneficial activities, such as anti-cancer[4,5], anti-oxidative[6], anti-inflammatory[7], antiobesity[8], anti-hypertensive[9], and memory recall properties[10], leading to a large number of studies on these plant-derived secondary metabolites. Traditionally, these compounds are mainly derived from plant extraction using organic solvents. However, due to their very low contents in plants[11-13], the production cost for most flavonols remains high, which imposes great restrictions on their application in healthcare and the food industry.

During the past decades, scientists have developed quite a number of methods to derive flavonoids[14,15]. However, chemical synthesis of these complicated molecules possesses a variety of intrinsic disadvantages[16]. It requires not only toxic reagents and extreme reaction conditions, but also many steps to produce a target flavonoid compound[14,17]. Moreover, another important challenge in this strategy is the chiral synthesis of active flavonoid molecules. Therefore, it is not an ideal strategy to produce flavonoids at a commercial scale via chemical synthesis[16,17].

Recently, scientists have developed a promising alternative strategy to produce these complicated natural compounds by engineering microbes with a pathway for flavonoid biosynthesis[18-22], which has been successfully deciphered in plants[23]. For example, Duan et al. introduced a biosynthetic pathway into the budding yeast *Saccharomyces cerevisiae* to produce kaempferol (KMF)[24]. Malla et al. produced astragalin, a glycosylated flavonol, by introducing flavanone 3-hydroxylase (*f3h*), flavonol synthase (*fls1*), and UDP-glucose:flavonoid 3-O-glucosyltransferase *UGT78K1* genes into *Escherichia coli* BL21(DE3)[17]. Even though there are quite a few paradigms, not all genetically engineered microbes produce the products of interest due to the complexity of a cellular platform, the incompatibility between artificially synthesized genetic elements and hosts, the inhibitory effect of target products against host cells, and the instability of an engineered cellular system itself[16].

Another promising alternative strategy for flavonoid production is to establish a multienzymatic cascade in vitro. Cheng et al. have reported that enterocin polyketides can be successfully synthesized by assembling a complete enzymatic pathway in one pot[25]. This cell-free synthetic strategy circumvents the restrictions of a microbial production factory and thus is feasible for producing some flavonoids in large quantity[16].

Recently, we have successfully developed a bienzyme synthetic system to convert naringenin (NRN) into KMF in one pot[16]. Here, we describe this system in great details and the methods involved in analyzing the products. We also present two examples that use this system to

produce KMF from NRN and quercetin (QRC) from eriodictyol (ERD). In addition, we discuss crucial steps of this method and future research directions in the biosynthesis of flavonoids.

PROTOCOL:

1. Isolate total RNA from plant tissues[26,27]

1.1) Homogenize the plant tissues.

1.1.1) Collect 100 mg of a fresh plant tissue (e.g., 4-week-old seedlings from *Arabidopsis thaliana*). Freeze the tissue and a pestle and mortar with liquid nitrogen, followed by grinding the tissue into powder.

1.1.2) Add 1 mL of RNA isolation reagent (see **Table of Materials**) into the mortar. The reagent will be frozen immediately. Homogenize the tissue sample with the pestle when the frozen reagent melts.

1.1.3) Transfer the homogenate to a 1.5-mL tube, centrifuge the sample at 12,000 x *g* for 5 min at 4 °C, and then transfer the cleared homogenate solution to another fresh 1.5-mL tube.

1.1.4) Incubate the homogenized sample at room temperature for 5 min.

1.2) Isolate total RNA.

1.2.1) Add 0.2 mL of chloroform to the homogenate, cap the tube securely, shake the tube vigorously by hand for 15 s, and incubate the sample at room temperature for 5 min.

1.2.2) Centrifuge the sample at 12,000 x *g* for 15 min at 4 °C and transfer the colorless upper aqueous phase to a fresh 1.5-mL tube. The sample separates into three phases following centrifugation.

1.2.3) Add 0.5 mL of isopropyl alcohol to the aqueous phase, shake the tube by hand in a vigorous manner, and incubate the mixture at room temperature for 10 min.

1.2.4) Centrifuge the mixture at 12,000 x *g* for 10 min at 4 °C and remove the supernatant.

1.2.5) Wash the RNA pellet once with 1 mL of 75% ethanol by vortexing, followed by centrifugation at 7,500 x *g* for 5 min at 4 °C.

1.2.6) Repeat step 1.2.5.

1.2.7) Air dry the pellet for 5-10 minutes and redissolve the RNA in diethylpyrocarbonate (DEPC)-treated water by pipetting up and down, followed by measuring the total RNA concentration with a micro-spectrophotometer (see **Table of Materials**).

2. Synthesize complementary DNA (cDNA)[28]

2.1) Synthesize the first strand of cDNA using a kit (see **Table of Materials**). Set up a 20-µL reaction system as shown in **Table 1** and incubate the reaction tube in a PCR instrument for 50 min at 42 °C, followed by terminating the reaction at 85 °C for 5 min. Store the reaction product at -20 °C for future amplification of genes.

[Place Table 1 here]

3. Construct recombinant plasmids[29]

3.1) Design PCR primers.

3.1.1) Design the PCR primers using a software (see **Table of Materials**) based on the sequences of key enzyme genes obtained from the GenBank database and synthesize the primers by a company (see **Table of Materials**). In the 5' end of the primer, add a restriction enzyme site (e.g., *Bam*HI or *Eco*RI in this protocol).

NOTE: The primers used in this study are shown in **Table 2**.

[Place Table 2 here]

3.2) Clone the genes into a prokaryotic expression vector.

3.2.1) Amplify the genes from the first strand of the synthesized cDNA using a high-fidelity DNA polymerase (see **Table of Materials**). Set up a 100-µL PCR reaction system as shown in **Table 3** and run the following PCR cycle: 94 °C for 2 min for initial denaturation; then 35 cycles of 94 °C for 30 s for denaturation, 55 °C for 2 min for annealing, and 72 °C for 1 min for extension; followed by a final elongation at 72 °C for 10 min. Cool the reaction mixture to 12 °C.

NOTE: The extension time is variable and determined by the gene length with polymerization of about 1000 bases per min for most DNA polymerases.

[Place Table 3 here]

3.2.2) Visualize the PCR products (most commonly 5 µL) on a 1% agarose gel and purify the specific DNA fragment from the remaining products using a DNA clean-up kit (see **Table of Materials**).

3.2.3) Digest the purified DNA fragment and the vector (e.g., pET-32a(+)) with restriction enzymes (e.g., *Bam*HI or *Eco*RI in this protocol). Set up a 50-µL reaction system in a 0.2 mL PCR tube as shown in **Table 4** and incubate the mixture at 37 °C for 3 h. Separate the digested DNA on a 1% agarose gel.

[Place Table 4 here]

3.2.4) Recover the DNA band using a gel extraction kit (see **Table of Materials**). Further purify the DNA using a DNA clean-up kit (see **Table of Materials**), followed by measuring the concentration of DNA with a micro-spectrophotometer (see **Table of Materials**).

3.2.5) Ligate the gene fragment into the linearized vector DNA using a T4 DNA ligase (see **Table of Materials**). Set up a ligation reaction in a 1.5-mL tube as shown in **Table 5** and incubate the tube at room temperature for 2 - 3 h.

NOTE: The molar ratio of an insert to a vector is variable and ranged from 3:1 to 10:1.

[Place Table 5 here]

3.2.6) Add 2.5 µL of the ligation mixture into 50 µL of chemically competent *Escherichia coli* cells (e.g., TOP10 or DH5α), mix gently, and keep the tube on ice for 30 min. Heat shock the cells at 42 °C for 90 s and immediately place the tube on ice for 2 min.

3.2.7) Add 200 µL of liquid LB medium without antibiotics into the tube and incubate the tube in a 37 °C shaker at 220 rpm for 1 h. Spread 50 - 100 µL of the cells on an LB plate containing 100 µg/mL ampicillin and incubate at 37 °C overnight.

3.3) Screen positive colonies.

3.3.1) Inoculate a single colony from the LB plate into 200 µL of liquid LB medium containing 100 µg/mL ampicillin and incubate at 37 °C, 250 rpm for 2 - 3 h.

NOTE: In general, pick 4 - 8 colonies for screening positive colonies.

3.3.2) Set up a 10-µL colony PCR reaction similar to that in step 3.2.1.

NOTE: Use 1 µL of LB culture instead of 1 µL of cDNA template.

3.3.3) Visualize the PCR products on a 1% agarose gel. Inoculate the remaining culture with a positive result into 3 mL of liquid LB medium containing 100 µg/mL ampicillin and incubate in a 37 °C shaker at 250 rpm for 14 - 16 h.

3.3.4) Isolate plasmid DNA from recombinant *E. coli* cultures using a plasmid miniprep kit (see **Table of Materials**).

3.3.5) Identify the purified recombinant plasmids by a double restriction enzyme analysis (e.g., *Bam*HI and *Eco*RI in this protocol). Set up a 10-µL reaction system similar to that in step 3.2.3, followed by incubation at 37 °C for 3 h. Visualize the specific band released from the

recombinant plasmid on a 1% agarose gel.

3.4) Verify the sequences of positive recombinant plasmids.

3.4.1) Send the plasmids to a company for sequencing. Analyze the results using a DNA sequence analysis software (see **Table of Materials**) by comparing the sequence obtained from the sequencing company with the reference sequence obtained from the GenBank database.

4. Express recombinant enzyme proteins[30]

4.1) Transform the correct recombinant plasmid into competent *E. coli* BL21(DE3).

4.1.1) Add 0.1 µL of the plasmid to 10 µL of competent *E. coli* BL21(DE3) in a 1.5-mL tube on ice and keep the tube on ice for 5 min.

4.1.2) Heat shock the cells in a 42 °C waterbath for 90 s and place it on ice again for 2 min.

4.1.3) Add 200 µL of LB liquid medium without antibiotics and incubate in a 37 °C shaker at 220 rpm for 5 min.

4.1.4) Spread 50 µL of transformation on an LB agar plate containing 100 µg/mL ampicillin and incubate the plate overnight in a 37 °C incubator.

4.2) Induce the expression of genes.

4.2.1) Inoculate 3 - 5 colonies from the plate into a tube containing 3 mL of LB liquid medium with 100 µg/mL ampicillin and incubate at 250 rpm in a 37 °C shaker overnight.

4.2.2) Transfer all of the overnight culture into 300 mL of LB liquid medium containing 100 µg/mL ampicillin and incubate at 250 rpm in a 37 °C shaker until the optical density of the culture at 600 nm is between 0.4 - 0.6.

4.2.3) Add isopropyl β-D-thiogalactoside (IPTG) into the culture with a final concentration of 0.2 mM and induce the expression of the genes at 250 rpm, 20 - 22 °C for 3 h.

5. Purify the recombinant enzyme proteins[31]

5.1) Harvest the bacteria by centrifugation of the culture at 4 °C, 12,000 x *g* for 10 min.

5.2) Resuspend the pellet in 15 mL of Bacterial Lysis Buffer containing 0.1% Triton X-100, 1 mM EDTA, 10% glycerol, 150 mM NaCl, 0.5 mM DTT, 0.1 mM PMSF, 1 µg/mL aprotinin, 1 µg/mL leupeptin, and 1 µg/mL pepstatin in 50 mM Tris-Cl (pH 8.0).

5.3) Sonicate the bacterial suspension to release the recombinant enzyme proteins, followed by

centrifugation at 13,000 x *g*, 4 °C for 10 min.

5.4) Harvest and aliquot the supernatant in 1.5-mL tubes at 1 mL/tube and store them at -70 °C for future use.

5.5) Apply 500 µL of His-tag purification resin (see **Table of Materials**) to a reusable empty affinity column. Wash the resin with 5 bed volumes of deionized water to discard the ethanol in the stock solution. Balance the resin with 10 bed volumes of the binding buffer comprising Tris-Cl (20 mM, pH 7.9), imidazole (10 mM), and NaCl (0.5 M).

5.6) Apply 4 mL of the supernatant from Step 5.4 to a slurry of the above resin and block two ends of the column with stoppers.

5.7) Incubate the mixture at 4 °C on a rotator at low speed for 2 h.

5.8) Wash the fusion protein bound resin with 15 bed volumes of the binding buffer at 4 °C at a flow rate of 1 mL/min before elution.

5.9) Add 500 µL of elution buffer (containing 20 mM Tris-Cl (pH 7.9), 500 mM imidazole, 0.5 M NaCl) to the column and incubate the slurry at 4 °C on a rotator at a low speed for 10 min. Collect the eluent as purified protein samples.

5.10) Repeat Step 5.5 four more times.

5.11) Wash the resin sequentially with 10 bed volumes of deionized water and 3 bed volumes of 20% ethanol. Soak the resin in 20% ethanol. Block the column with stoppers and store it at 4 °C.

5.12) Measure the concentration of the purified proteins by the Bradford protein assay. Determine the purity of the proteins on a 10% SDS-PAGE gel and visualize the bands by the Coomassie blue staining assay.

5.13) Add glycerol to the purified protein solution to a final concentration of 10% to stabilize the enzyme activity. Aliquot and store it at -80 °C.

6. Produce a flavonol from a flavanone in an in vitro bienzyme synthetic system[16]

6.1) Prepare buffers.

6.1.1) Make 2x synthetic buffer without ferrous sulfate consisting of 200 mM Tris-HCl (pH 7.2), 16.4 mM α-ketoglutaric acid, 0.8% sodium ascorbate, and 20% glycerol. Dissolve 0.969 g of Tris base, 0.320 g of sodium ascorbate, 0.125 g of α-ketoglutaric acid, and 8 mL of glycerol to 32 mL of deionized water. Adjust pH to 7.2 by hydrochloric acid (HCl) and add deionized water up to 40 mL. Store the buffer at 4 °C for future use.

6.1.2) Make a 100x stock solution of 2 mM ferrous sulfate. Dissolve 55.6 mg of ferrous sulfate heptahydrate in 50 mL of deionized water, stir, and add water up to 100 mL.

6.1.3) Make a stock solution of 25 mM flavonoid. Dissolve a flavonoid in methanol thoroughly and stored at -20 ° C.

6.2) Set up a synthetic system to produce a flavonol from a flavanone.

6.2.1) Prepare the synthetic system as shown in **Table 6**.

[Place Table 6 here]

6.2.2) Incubate the reaction at 40 °C in an open 2.0-mL tube at 600 rpm (in a shaking heat block) for 40 min.

6.2.3) Terminate the reaction by adding 10 µL of acetic acid and 100 µL of ethyl acetate.

6.2.4) Two hours later, transfer the organic phases to 1.5-mL tubes for drying in a vacuum freeze-drying system (see **Table of Materials**).

7. Analyze the reaction products

7.1) Thin layer chromatography (TLC) analysis.

7.1.1) Redissolve the flavonoid powder from step 6.2.4 in 100 µL of methanol. Prepare authentic flavonoid samples with serial concentrations of 12.5, 25, 50, 100, and 200 ng/µL in methanol. Load 1 µL of the reaction samples and the authentic flavonoid samples onto polyamide 6 plates.

7.1.2) Run the sample-loaded plates in a solvent system comprising chloroform/methanol/ethyl acetate/formic acid at a ratio of 5.0:1.5:1.0:0.5.

7.1.3) Air dry the plates at room temperature. Spray the plates with 1% ethanolic solution of aluminum chloride ($AlCl_3$), followed by air drying again at room temperature.

7.1.4) Thirty minutes later, visualize the spots on the plates under a UV light at 254 nm and take images.

7.1.5) Analyze the gray value of each spot on the images using an image processing software (e.g., ImageJ v1.51j8 in this protocol).

7.1.5.1) Open the software ImageJ. Click **File > Open** to open the image to be analyzed.

7.1.5.2) Click the left most **Rectangular Selection Tool** in the ImageJ User Interface. Outline the region of interest (ROI) in the image with the mouse and press [1] to label the first ROI.

7.1.5.3) Move the rectangular selection with the mouse right to the next ROI and press [2] to label the second ROI.

7.1.5.4) Repeat the previous step to label all other ROIs.

7.1.5.5) Press [3] to generate profile plots for all ROIs in a pop-up window.

NOTE: At this time, the **Straight Line Selection Tool** in the ImageJ User Interface will be automatically activated.

7.1.5.6) Use the **Straight Line Selection Tool** to draw base lines so as to define a closed area for each peak of interest.

7.1.5.7) Activate the **Wand Tool** by clicking the corresponding icon in the ImageJ User Interface. Click inside the peak to display results for all peaks in a pop-up window.

7.1.6) Make a TLC-based standard curve of the authentic flavonoid by plotting the gray values from step 7.1.5.7 against the corresponding flavonoid concentrations from step 7.1.1. Then, calculate the yield of the flavonoid of interest produced in this protocol according to the resulting formula.

7.2) High performance liquid chromatography (HPLC) and liquid chromatography/mass spectrometry (LC/MS) analyses

7.2.1) Pool 5 tubes of the flavonoid samples from step 6.2.4 and take out 300 µL for drying. Redissolve the powder in 160 µL of methanol. Prepare authentic flavonoid samples with serial concentrations of 20, 40, 60, 80, and 100 ng/µL in methanol. Process the samples sequentially through 0.45 µm and 0.22 µm filters.

7.2.2) Load the samples into a HPLC/LC/MS system (see **Table of Materials**) and separate the samples at 30 °C using a C18 (4.6 × 150 mm; i.d., 5 µm) column. Elute the column at 1.0 mL/min by a gradient of 10 - 85% (v/v) acetonitrile (ACN) in water (0 - 10 min, 10 - 25% ACN; 10 - 35 min, 25 - 50% ACN; 35 - 45 min, 50 - 85% ACN; 45 - 50 min, 85 - 10% ACN; 50 - 60 min, 10% ACN) and monitor the absorbance of the eluate from 200 to 800 nm. Perform the LC/MS analysis in a negative ion mode with a drying nitrogen flow of 10 L/min at 300 °C and a sheath gas flow of 7 L/min at 250 °C and collect data using a built-in software (see **Table of Materials**).

7.2.3) Extract single wavelength chromatographs to calculate the peak areas of reaction samples and authentic flavonoid compounds using a software (see **Table of Materials**).

7.2.3.1) Open the Qualitative Analysis program and click **File > Open Data File.** Select the file(s) to be analyzed in the **Open Data File** window and click **Open** to open the file(s).

7.2.3.2) Right-click the mouse in the **Chromatogram Results** window and then the **Extract Chromatograms** in a pop-up menu.

7.2.3.3) Open the **Extract Chromatograms** dialog box. In the **Type** list, click **Other Chromatograms**. In the **Detector** combo box, select DAD1. Then click **OK** to display the HPLC results in the **Chromatogram Results** window.

7.2.3.4) Click the **Manual Integration** icon docked at the top of the **Chromatogram Results** window. Draw a base line for the peak required for manual integration analysis with the mouse.

7.2.3.5) Click **View > Integration Peak List** to display the results.

7.2.4) Make a HPLC-based standard curve of the authentic flavonoid by plotting the peak areas from step 7.2.3.5 against the corresponding flavonoid concentrations from step 7.2.1. Then, calculate the yield of the flavonoid of interest produced in this protocol according to the resulting formula.

7.2.5) Analyze the MS data for the exact mass of flavonoid compounds using a software (see **Table of Materials**).

7.2.5.1) Repeat steps 7.2.3.1 - 7.2.3.3.

7.2.5.2) Click the **Range Select** icon on the **Chromatogram Results** toolbar.

7.2.5.3) Select the peak of interest. Right-click the mouse in the selected range and click the **Extract MS Spectrum** in the pop-up menu to display the results in the **MS Spectrum Results** window.

REPRESENTATIVE RESULTS:

F3H and FLS1 are two important key enzymes in the conversion of a flavanone into a flavonol in plants as shown in **Figure 1**. To develop an in vitro biosynthetic system for producing a flavonol from a flavanone, *Atf3h* (GenBank accession no. NM_114983.3) and *Atfls1* (GenBank accession no. NM_120951.3) genes were cloned from the seedlings of 4-week-old *A. thaliana* into a prokaryotic expression vector pET-32a(+). The recombinant plasmids were transformed into *E. coli* BL21(DE3) and the fusion proteins were expressed after IPTG induction, followed by purification using Ni-IDA agarose resins. As shown in **Figure 2**, the purified fusion proteins showed a high purity of over 95% on a 10% SDS-PAGE gel, which were pure enough for the establishment of an in vitro bienzymatic cascade.

[Place Figure 1 here]

[Place Figure 2 here]

To establish a bienzymatic cascade using the purified recombinant proteins, a synthetic system was prepared as shown in **Table 6**. To determine whether this system can be used for the conversion of a flavanone into a flavonol, NRN was added into the system, and the biosynthesis of KMF was detected by TLC and HPLC/LC/MS analyses. As shown in **Figure 3A**, there were two new spots emerged on a polyamide TLC plate. One spot showed a migration distance similar to that of dihydrokaempferol (DHK), and the other similar to that of KMF. Further analysis by HPLC and LC/MS demonstrated that the new chemicals showed a retention time of 11.91 min and 20.16 min, respectively (**Figure 3B**) and a quasi-molecular ion peak $[M-H]^-$ at m/z 287.0500 and 285.0500, respectively (**Figure 3C**), which were identical to those of DHK and KMF, respectively. The data indicate that KMF was produced from NRN in this system and the yield was as high as 34.94 mg/L.

[Place Figure 3 here]

To further determine whether this in vitro system can be used for the conversion of other flavanones into their corresponding flavonols, eriodictyol (ERD) was added into the system to determine whether ERD can be converted into quercetin (QRC). As shown in **Figure 4A**, two new spots on a polyamide TLC plate displayed a migration distance similar to that of dihydroquercetin (DHQ) and QRC, respectively. HPLC and LC/MS analyses demonstrated that these new chemicals revealed a retention time of 10.03 min and 16.23 min, respectively (Fig**ure 4B**) and a quasi-molecular ion peak $[M-H]^-$ at m/z 303.1000 and 301.1000, respectively (**Figure 4C**), which exactly corresponded to those of DHQ and QRC, respectively. The data indicate that this system can convert ERD into QRC and the yield was 25.55 mg/L.

[Place Figure 4 here]

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic representation for the biosynthesis of a flavonol from a flavanone in vitro**.** F3H, flavanone 3-hydroxylase; FLS1, flavonol synthase 1.

Figure 2: Purification of recombinant AtF3H and AtFLS1 proteins. The *Atf3h* and *Atfls1* genes were cloned from 4-week-old seedlings of *Arabidopsis thaliana* into a prokaryotic expression vector pET-32a(+) and expressed in *Escherichia coli* BL21(DE3). The recombinant proteins were purified through an affinity chromatography column filled with Ni-IDA agarose resins. The purity was determined on a 10% SDS-PAGE gel. M, protein markers; 1, recombinant AtF3H protein; 2, recombinant AtFLS1 protein.

Figure 3: Synthesis of KMF from NRN in a bienzymatic cascade. (**A**) Analysis of the one-pot reaction products by polyamide TLC. 1, NRN standard; 2, DHK standard; 3, KMF standard; 4, reaction mixture. (**B**) HPLC analysis profiles of the reaction products. NRN, DHK, and KMF showed a retention time of 18.74 min, 11.91 min, and 20.16 min, respectively. (**C**) MS analysis

profiles of the flavonoid compounds in the reaction mixtures.

Figure 4: Production of QRC from ERD in a bienzyme synthetic system. (**A**) Analysis of the reaction products by polyamide TLC. 1, ERD standard; 2, DHQ standard; 3, QRC standard; 4, reaction mixture. (**B**) HPLC analysis profiles of the reaction products. ERD, DHQ, and QRD displayed a retention time of 15.45 min, 10.03 min, and 16.23 min, respectively. (**C**) MS analysis profiles of the compounds in the reaction mixtures.

Table 1: Reverse transcription of total RNA into cDNA

Table 2: Oligonucleotide primers used in the current study

Table 3: Setting up of a PCR reaction system

Table 4: Double digestion of a DNA fragment/vector

Table 5: Ligation of a gene fragment into a linearized vector

Table 6: The synthetic system used in this protocol.

DISCUSSION:

Quite a number of studies are focused on the derivation of flavonols due to their potential application in health care and food industry. However, traditional plant extraction using organic solvents and chemical synthesis possess intrinsic disadvantages, which restrict their use in the production of flavonols. Here, we report a detailed method for producing a flavonol from a flavanone in one pot by establishing an in vitro bienzymatic cascade. The critical steps in this protocol are: 1) obtaining pure recombinant enzymes with high activities and 2) establishing a one-pot bienzymatic reaction cascade. Generally speaking, the expression of plant-derived genes in bacteria prefers to form inclusion body, which will lead to the loss of enzyme activity. As we know, some peptides, such as TrxA and SUMO, help to enhance the expression and solubility of recombinant proteins expressed in bacteria[16]. Therefore, it will be helpful to clone the target genes into the plasmids containing these expression tags, such as pET-32a(+) and pET SUMO (Step 3.2.3). It is well known that IPTG concentration and induction temperature are another two crucial parameters affecting the solubility of prokaryotically expressed proteins[16]. To further decrease the formation of inclusion body, IPTG concentration and induction temperature should be optimized. The optimum IPTG concentration and induction temperature mainly depends on the type of plasmids and the bacteria strains. In this protocol, the IPTG concentration and induction temperature are optimized at 0.2 mM and 20 - 22 °C, respectively (Step 4.2.3). In addition, temperature and glycerol are two important parameters for maintaining the stability and activity when purifying and storing the recombinant enzymes. In this protocol, it is crucial to purify the recombinant proteins at 4 °C (Steps 5.5 - 5.12), add 10% glycerol into the solution of purified enzymes (Step 5.13), and immediately aliquot and store the solution at -80 °C (Step 5.13). In establishment of a one-pot reaction cascade, pH and temperature are two vital parameters. It is obvious that too high pH is harmful for the

conversion because the ferrous ions (Fe^{2+}), a necessary component for the enzyme activity of recombinant F3H and FLS1[16,32,33], are precipitated by forming a slurry of ferrous hydroxide under such a condition. Even though a relatively higher temperature facilitates the progress of an enzyme-catalyzed reaction, too high temperature will inactivate the enzyme. Therefore, it is critical for the conversion to stabilize the pH and reaction temperature. Our previous publication sets the optimum pH and temperature at 7.2 and 40 °C, respectively (Step 6)[16].

This protocol could be conveniently modified to biosynthesize a number of flavonols from various flavanones using different substrates. In this protocol, two examples are provided. As shown in **Figure 3**, when adding NRN as a substrate into this system, new chemicals were produced. TLC and HPLC/LC/MS analyses indicate that the new chemicals were DHK and KMF, and the NRN was converted into the KMF in this system. To further strengthen confidence in the results, spectral characterization of ^{1}H NMR (hydrogen-1 nuclear magnetic resonance), ^{13}C NMR (carbon-13 nuclear magnetic resonance), NOESY (Nuclear Overhauser Effect SpectroscopY), XRD (X-ray powder diffraction), CHN analyzer and the like may be required to attest the presence of chemicals in a new entity. Similarly, ERD could be successfully converted into QRC in this bienzymatic cascade (**Figure 4**). The yield of the KMF was higher than that of the QRC, indicating that the NRN is a better substance for this flavonol production system than the ERD.

There is an important limitation for this method. According to the known biosynthetic pathway of flavonoids, a flavonol can be produced by this system from an aromatic amino acid or its downstream derivatives. For example, KMF can be produced from *p*-coumaric acid by a series of key enzymes, including 4-coumaroyl:CoA-ligase (4CL), chalcone synthase (CHS), chalcone isomerase (CHI), F3H and FLS[23]. Similarly, QRC can be produced from caffeic acid using the same panel of key enzymes (unpublished data). However, coenzyme A (CoA), ATP, and manonyl-CoA need to be included in the system to convert *p*-coumaric acid into KMF, which will greatly increase the production cost. Therefore, this system is usually restricted to convert a flavanone into a dihydroflavonol or a flavonol. In addition, complete conversion of starting materials is another challenge. To further improve the efficiency of this system, future research should be focused on screening key enzymes with high activities from other plants, mutation of genes encoding key enzymes, immobilization of the highly active enzymes to inert carriers, and development of a better buffer system.

This one-pot bienzyme synthetic system possesses obvious intrinsic advantages over other approaches to produce a flavonol, such as chemical synthesis, microbial cell factory, and plant extraction using organic solvents[16]. Firstly, the reaction time is very short and needs only 40 min, so this production system is labor- and time-saving. Secondly, there is no complex physiological regulation in this system as occurred in the microbial cell factory and moreover, all components are clear. Therefore, it is easy to control the reaction accurately and thus convenient to make further optimization in the future. Thirdly, since this reaction system contains only simple chemicals and purified recombinant enzymes and only generates one major intermediate as shown in **Figure 3** and **Figure 4**, it is expected that it is easier to purify the target molecules generated in this system than those from cell factories and plants. Fourthly, the major

components of the system are common and cheap chemicals and prokaryotically expressed recombinant enzymes, so it is highly cost-effective for this method to derive desired flavonoids. Fifthly, due to the simplicity of the components of this system, it is easy to scale up for mass production of target flavonoids, indicating a huge industrialization potential. In addition, this system provides a guide for the economical production of other secondary metabolites.

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DISCLOSURES:

The authors declare that they have no competing financial interests.

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Figure 1

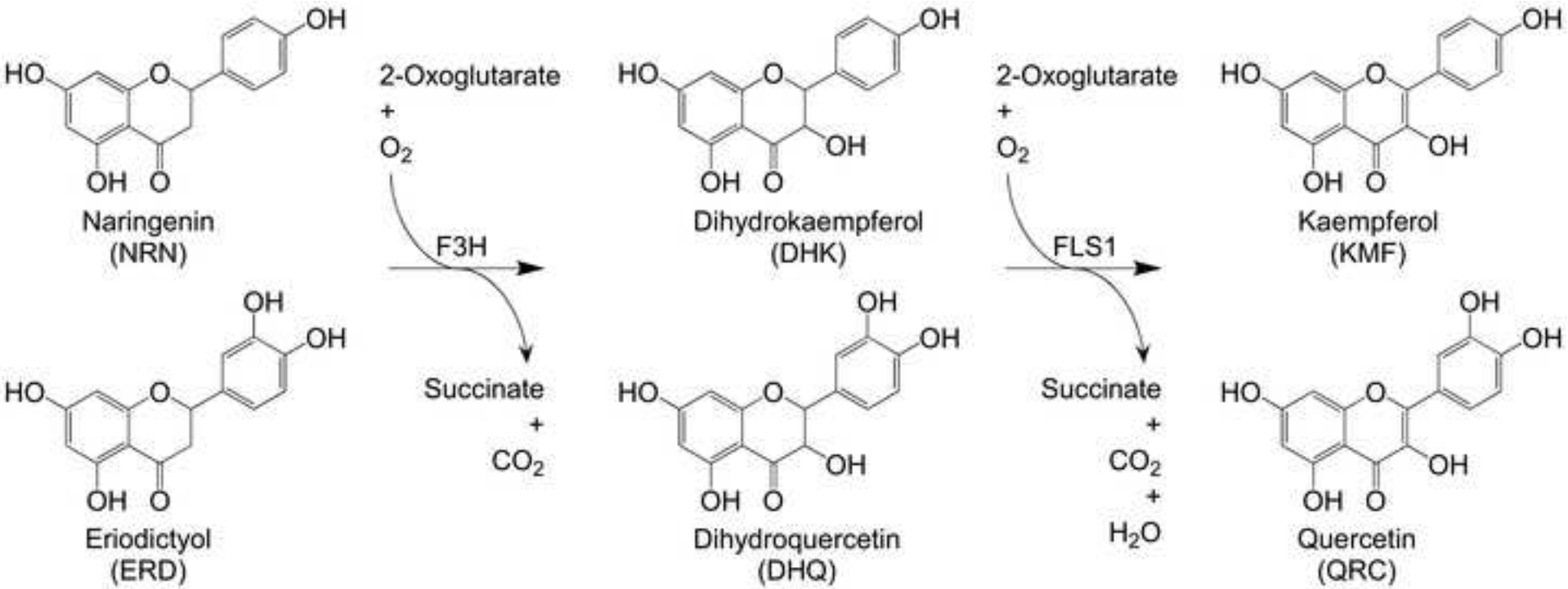

Figure 3

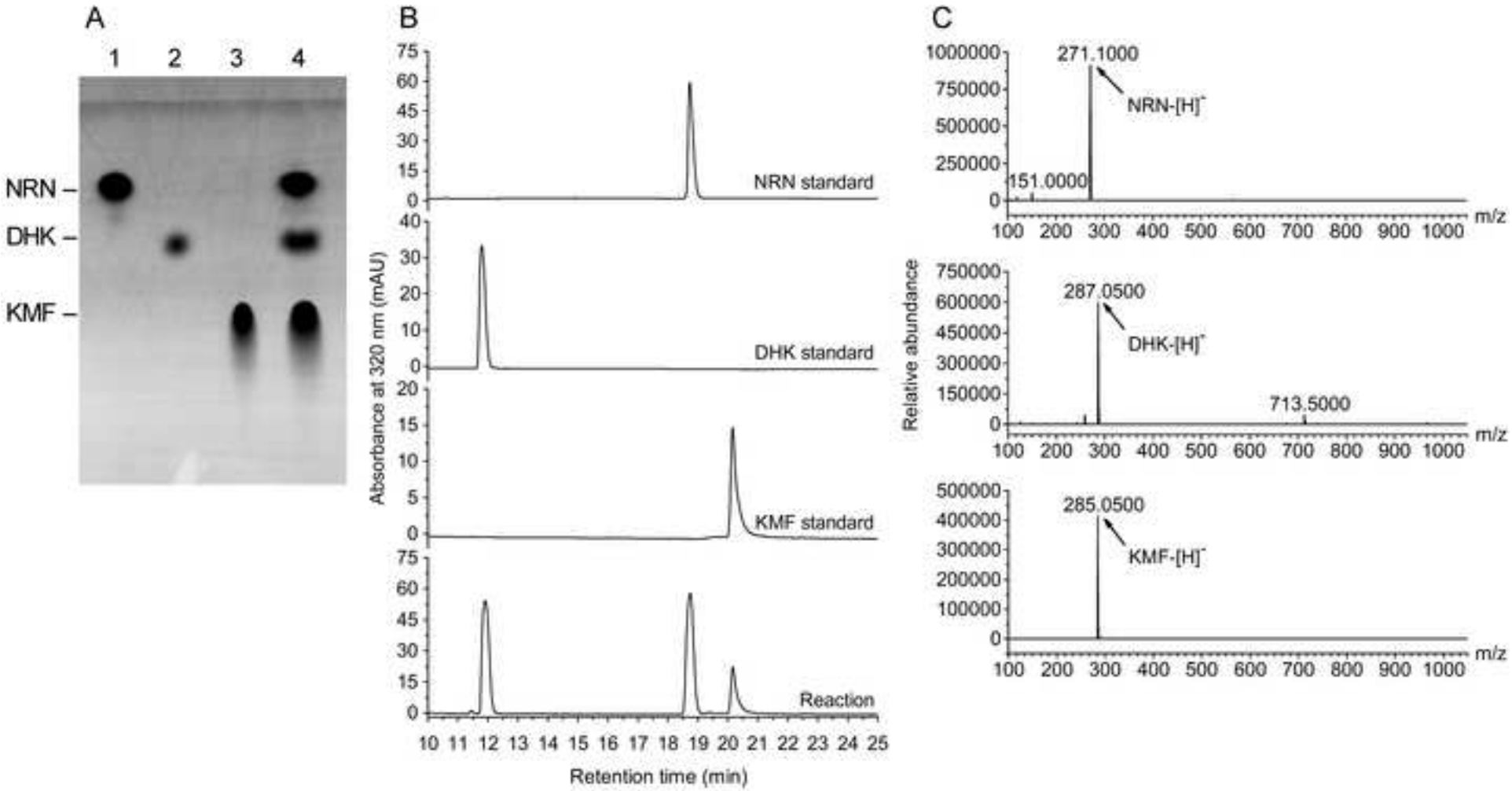

Figure 4

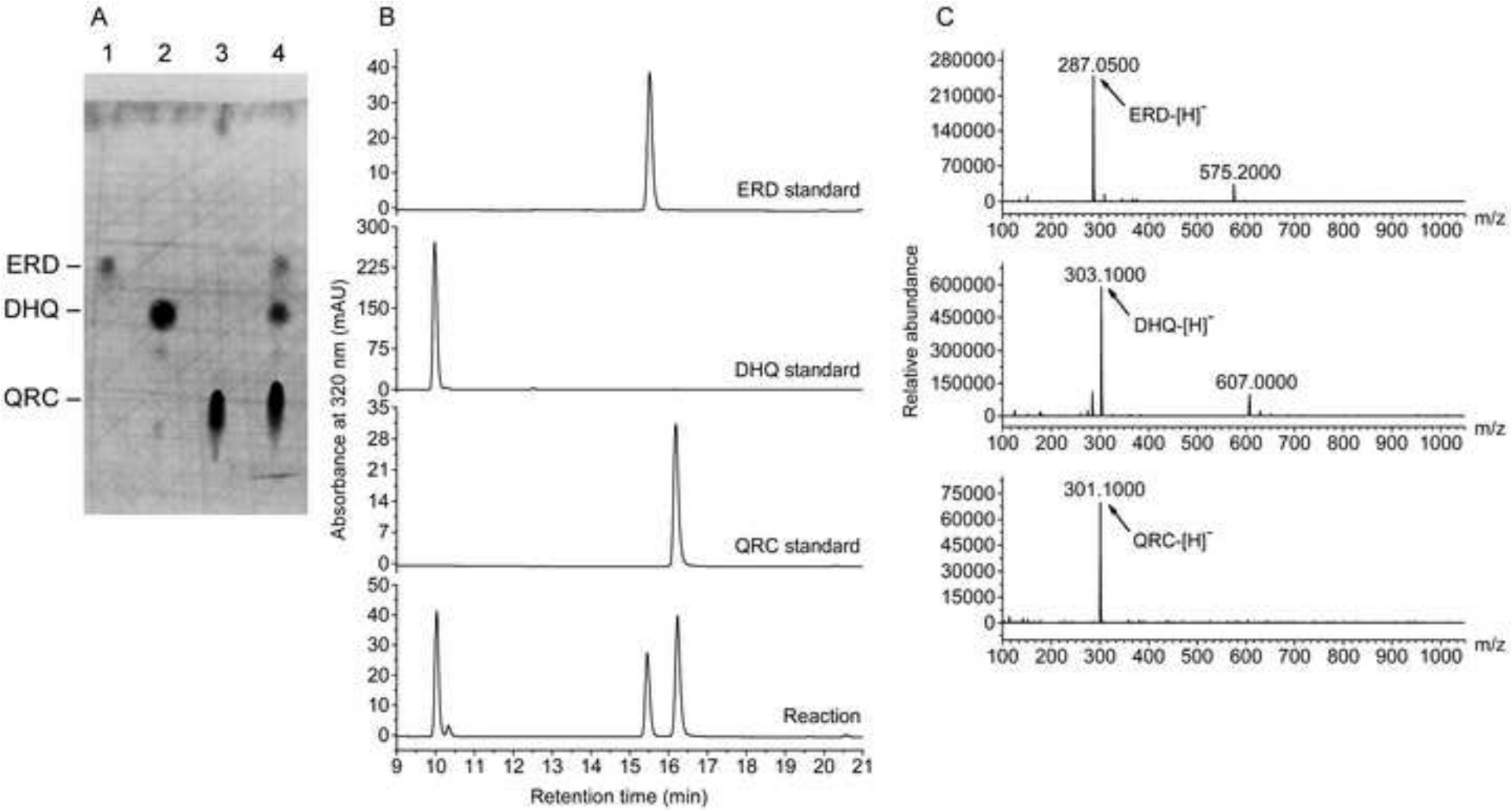

Table 1 Reverse transcription of total RNA into cDNA

Reagents	Volume
dNTP Mix, 2.5mM each	4.0 µL
Primer Mix	2.0 µL
RNA Template	1.0 µg
Reverse Transcriptase Buffer, 5×	4.0 µL
Reverse Transcriptase, 200 U/µL	1.0 µL
RNase-Free H_2O	up to 20.0 µL

Table 2 Oligonucleotide primers used in the current study

Sequence, 5' → 3'	Purpose
AA*GGATCC* ATGGCTCCAGGAACTTTGACT	Forward primer for PCR amplification of *Atf3h* gene from *Arabidopsis thaliana.* *Bam* HI site is italicized and attached for cloning into pET32a(+).
AA*GAATTC* CTAAGCGAAGATTTGGTCGA	Reverse primer for PCR amplification of *Atf3h* gene from *A. thaliana.* *Eco* RI site is italicized and attached for cloning into pET32a(+).
AA*GGATCC* ATGGAGGTCGAAAGAGTCCA	Forward primer for PCR amplification of *Atfls1* gene from *A. thaliana.* *Bam* HI site is italicized and attached for cloning into pET32a(+).
AA*GAATTC* TCAATCCAGAGGAAGTTTAT	Reverse primer for PCR amplification of *Atfls1* gene from *A. thaliana.* *Eco* RI site is italicized and attached for cloning into pET32a(+).

Table 3 Setting up of a PCR reaction system

Reagents	Volume
Pfu Master Mix, 2×	50.0 μL
Forward Primer, 10 μM	4.0 μL
Reverse Primer, 10 μM	4.0 μL
cDNA	2.0 μL
H_2O	40.0 μL

Table 4 Double digestion of a DNA fragment/vector

Reagents	Volume
DNA Fragment/Vector	3.0 μg
Bam HI	1.0 μL
Eco RI	1.0 μL
Cutsmart Buffer, 10×	5.0 μL
H_2O	up to 50.0 μL

Table 5 Ligation of a gene fragment into a linearized vector

Reagents	Volume
Insert	X µL (0.09 pmol)
Vector	Y µL (0.03 pmol)
Ligation Buffer, 10×	1.0 µL
T4 DNA Ligase, 400 U/µL	1.0 µL
H_2O	up to 10.0 µL

Table 6 Setting up of an in vitro synthetic system for the production of a flavonol from a flavanone

Reagents	Volume
2× Synthetic Buffer without ferrous sulfate	50.0 µL
25 mM flavonol	2.0 µL
2 mM ferrous sulfate	0.5 µL
1 mg/mL AtF3H	2.5 µL
1 mg/mL AtFLS1	2.5 µL
25 mM flavanone	2.0 µL
H_2O	up to 100.0 µL

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
2× Pfu MasterMix	Beijing CoWin Biotech Co., Ltd	CW0717A	PCR amplification of genes with high fidelity
Agilent 1200 Series RRLC system with an Agilent 6460 Triple Quadrupole LC/MS system	Agilent Technologies, Inc	N/A	an equipment for analysis of flavonoids by HPLC/MS
Agilent MassHunter Workstation (version B.03.01)	Agilent Technologies, Inc	N/A	a software for collection of the data from the Agilent 1200 Series RRLC system with an Agilent 6460 Triple Quadrupole LC/MS system
dihydrokaempferol	Sigma-Aldrich Co. LLC	91216	intermediate product for producing kaempferol from naringenin
dihydroquercetin	Sichuan Provincial Standard Substance Center for Chinese Herbal Medicine	PCS0371	intermediate product for producing quercetin from eriodictyol
DNA Clean-up Kit	Beijing CoWin Biotech Co., Ltd	CW2301	purification of PCR-amplified or gel-purified DNA
eriodictyol	Shanghai Yuan Ye Biotechnology Co., Ltd.	B21160	substrate for producing quercetin
Escherichia coli BL21(DE3)	Beijing CoWin Biotech Co., Ltd	CW0809	bacteria strain for expressing target genes
Escherichia coli DH5α	Beijing CoWin Biotech Co., Ltd	CW0808	bacteria strain for plasmid proliferation
FreeZone 1 Liter Benchtop Freeze-Dry System	Labconco Corporation	7740020	an equipment for freeze-drying of flavonoids dissolved in organic solvent
Gel Extraction Kit	Beijing CoWin Biotech Co., Ltd	CW2302	purification of a DNA band from an agarose gel

Gel Imaging System	Shanghai Tanon Science & Technology Co. Ltd.	Tanon-2500	an equipment for visualization of DNA band on an agarose gel or flavonoid spot on a polyamide TLC plate
GenElute Plasmid Miniprep Kit	Sigma-Aldrich Co. LLC	PLN350-1KT	minipreparation of plasmids
kaempferol	Sigma-Aldrich Co. LLC	60010	final reaction product and standard substance
MassHunter Quanlitative Analysis (version B.01.04)	Agilent Technologies, Inc	N/A	a software for analysis of HPLC/LC/MS data
NanoDrop Microvolume UV-Vis Spectrophotometer	Thermo Fisher Scientific	ND-8000-GL	an equipment for determination of DNA/RNA concentration
naringenin	Sigma-Aldrich Co. LLC	N5893	substrate for producing kaempferol
Ni-IDA Agarose Resin	Beijing CoWin Biotech Co., Ltd	CW0010	purification of His-tagged fusion proteins
pET-32a(+)	Novagen	69015-3	plasmid for cloning and expressing target genes
plasmid sequencing	GENEWIZ Suzhou	N/A	sequencing of recombinant plasmids
primer synthesis	GENEWIZ Suzhou	N/A	synthesis of PCR primers
quercetin	Shanghai Aladdin Biochemical Technology Co.,Ltd.	Q111273	final reaction product and standard substance
SuperRT cDNA Synthesis Kit	Beijing CoWin Biotech Co., Ltd	CW0741	synthesis of the first strand of cDNA from total RNA
T4 DNA Ligase	Thermo Fisher Scientific	EL0016	ligation of an insert into a linearized vector DNA
Trizol	Thermo Fisher Scientific	15596018	isolation of total RNA
Vector NTI Advance	Thermo Fisher Scientific	12605099	a software for PCR primer design and DNA sequence analysis
Xcalibur v2.0.7	Thermo Fisher Scientific	N/A	a software for analysis of HPLC data

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Dear Editor,

Thanks for your interest in our manuscript. Followed are our responses to all editorial and reviewers' comments.

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Our response: We have thoroughly proofread the manuscript.

2. Please revise the title to be more concise. For instance, "A method for the" may be deleted.

Our response: We have revised the title as you suggested.

3. Please provide an institutional email address, if possible, for each author.

Our response: Yes, we have provided an institutional email address for each author.

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Trizol, NanoDrop, Thermo Scientific, Beijing CoWin Biotech Co., Ltd., Vector NTI Advance, GENEWIZ, Vazyme Biotech Co., Ltd, GenElute, Sigma-Aldrich Co. LLC, Agilent, Labconco, etc.

Our response: We have replaced all commercial language with generic terms.

5. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.

Our response: We have added enough details to all protocol steps and added related references to published materials.

6. For PCR, please specify PCR primers and conditions throughout the protocol.

Our response: The PCR primers are specified in step 3.1.1 and Table 2. The PCR conditions are specified in step 3.2.1.

7. 3.1: Please note that the design step 3.1.1 is not appropriate for filming.

Our response: The step 3.1.1 is rewritten and also excluded from filming in the revision.

8. 3.2.1-3.2.4: if these steps are included for filming, specific details about how to perform these steps are required. Referring to the manufacturer's manual only is not sufficient.

Our response: We rewrite 3.2.1-3.2.4, but don't plan to film this part because it is a routine experiment.

9. 6.2.4: Are the organic phases frozen before drying in a freeze dryer?
Our response: No.

10. 7.1.1: Please provide some guidance on the appropriate volume of methanol.
Our response: We rewrite this part in the revision.

11. 7.1.4: Please describe how to analyze the gray value using ImageJ.
Our response: We have described it in details in step 7.1.5 in the revision.

12. 7.1.5, 7.2.3: Please describe how. Software steps must be more explicitly explained ('click', 'select', etc.). Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc.).
Our response: We have described these two parts in details in the revision.

13. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
Our response: Yea, we did it.

14. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.
Our response: Yes, we have highlighted all complete sentences critical for filming.

15. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.
Our response: Yes, we did so.

16. Figure 3A and Figure 4A: Please mark the fragment sizes.
Our response: These figures are TLC results and we indicate the identity of the dots in the image.

17. References: Please do not abbreviate journal titles.
Our response: We have corrected all abbreviated journal titles.

18. Table of Materials: Please sort the items in alphabetical order according to the name of material/equipment.
Our response: We sorted the items in alphabetical order according to the name of material/equipment.

Reviewers' comments:

Reviewer #1:

1. Though it is mentioned in the "Discussion part" as "TLC and HPLC-MS analyses indicate that the new chemicals were DHK and KMF", may I add my opinion in this regard as "Spectral characterization of 1HNMR, 13CNMR, NOESY, XRD, CHN analysis and the like many required to attest the presence of chemicals in a new entity". Though not all, may I suggest as few can be studied to characterize new entity chemicals.

Our response: We have added this sentence in the second paragraph of the Discussion part (Lines 535-539).

2. In the Protocol, say in the procedures of all seven topics, viz., i) Isolation RNA ii) Synthesizing CDNA and so on ------- to vii) Analyze the reaction products, May I Know a fact that all steps written in all seven procedures are authors' if not, then, no references being mentioned anywhere in all seven steps of protocol.

Our response: Actually, we write the procedures of all seven procedures. In the revision, we cited the related publications.

3. From the scheme, it is clear that flavonol is being produced from flavanone, but many in other paras like in introduction part or in representative results or in Discussion, then and there, somewhere, it was found to be written as though flavanone being produced from flavanol.

Our response: We correct the typo.

Reviewer #2:

Manuscript Summary:

A method for the in vitro enzymatic synthesis of flavonol was developed, and two enzymes were expressed by the corresponding Atf3h and Atfls1 genes and used to catalyze the synthesis of flavonol in vitro. The method displays obvious advantages for synthesis of natural products. However, in this work some problems remained unclear and indistinct such as something in Figure 1. In the second part, DHK and DHQ were catalyzed to form the KMF and QRC by dehydrogenation, respectively. In this reaction, where is the hydrogen acceptor? In addition, the paper was not carefully prepared and there were many mistakes in the manuscript. In the Reference, the names of authors were not listed completely consistent according to the requirements of JoVE. Some headlines were given as verb phrase such as that in page 2.

Our response: Figure 1 was a simplified schematic representation and only key substrate was shown. In the revision, we have modified Figure 1 with more details showing that the hydrogen acceptor is O_2. In addition, we have carefully revised the manuscript and corrected all mistakes we can find including that in the Reference part.

Reviewer #3:

There is no question required to address.

Reviewer #4:

1. 7.1.1) Redissolve the flavonoid powder from step 6.2.4 in an appropriate volume of methanol." It should be more precisely described.

Our response: We describe it more precisely in the revision.

2. 7.2.2). " Elute the column at 1.0 mL/min by a 10-85% (v/v) gradient of acetonitrile in water (0-10 min, a linear gradient of 10-25% (v/v) B; 10-35 min, a linear gradient of 25-50% B in 75-50% A; 35-45 min, a linear gradient of 50-85% B in 50-15% A; 45-50 min, a linear gradient of 85-10% B in 15-90% A; 50-60 min, 10% B in 95% A) and monitor the absorbance of the eluate from 200 to 800 nm." The description of gradient program of the mobile phase based on the reporting the changes of B component are enough. It should be corrected to the form presented above.
Our response: We have corrected the description of the gradient program in step 7.2.2.

3. References. The abbreviations of the names of journals in positions 2 and13 should be used.
Our response: We have corrected all errors in the Reference part.

4. Minor editorial mistake.
In the manuscript there are some editorial mistakes such as e.g.
Page 2 line 143: 20μL
Page 5 line 236: (pH8.0)
Page 5 line 249: pH7.9),
Page 5 line 260: (pH7.9),
Page 6 line 287: FeSO4·7H2O
Our response: We correct all these mistakes in the revision.

Reviewer #5:
Major Concerns:
Yield and purity Calculations. HPLC standard curve is not discussed in results. Side products and complete conversion of starting material.
Our response: We describe the calculation of the yield and the HPLC standard curve in details in the revision. We also discuss the side products and complete conversion of starting material in the Discussion part of the revision. But, we do not mention purity calculation because this protocol is focused on generation of a system for production of a flavonol from a flavanone, not for purification of a flavonol.

We are looking forward to hearing further information from you.

Sincerely yours,

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