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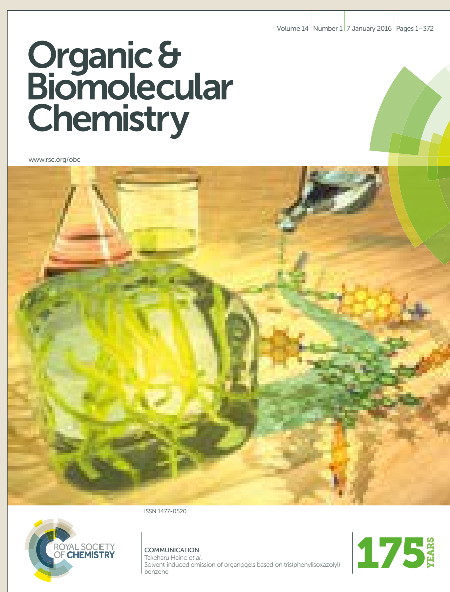

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A regiospecific rhamnosyltransferase from *Epimedium pseudowushanense* catalyzes the 3-*O*-rhamnosylation of prenylflavonols

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Epimedium is used in traditional Chinese medicine and contains flavonol glycosides that exhibit multiple biological activities. These bioactive flavonol glycosides usually have a rhamnose moiety at the 3-OH position of prenylflavonols, such as icariin (**9**), baohuoside I (**1a**) and baohuoside II (**2a**). However, to date, no rhamnosyltransferase has been reported to catalyze the 3-*O*-rhamnosylation of prenylflavonols. In this article, a flavonol rhamnosyltransferase, EpPF3RT, was identified from *E. pseudowushanense* B.L.Guo. The recombinant enzyme regiospecifically transfers a rhamnose moiety to 8-prenylkaemferol (**1**) and anhydroicaritin (**2**) at the 3-OH position to form baohuoside II (**1a**) and baohuoside I (**2a**) *in vitro*. In addition, a UDP-rhamnose synthase gene, *EpRhS*, from *E. pseudowushanense* was functionally characterized and used to produce the UDP-rhamnose sugar donor. Furthermore, an engineered *Escherichia coli* strain containing *EpPF3RT* and *EpRhS* was established to produce baohuoside II (**1a**) from whole cells. These studies indicate the significant potential of an enzymatic approach for the rhamnosylation of bioactive flavonoids in *Epimedium* plants and will provide a promising alternative for producing bioactive rhamnosylated flavonoids combined with other genes/enzymes by synthetic biology.

Introduction

Epimedium has been used in traditional Chinese medicine to invigorate the kidneys and strengthen muscles and bones. It also possesses multiple biological activities like anti-oxidant, anti-inflammatory, anti-osteoporotic, anti-cancer, neuroprotective, angiogenesis stimulating, testosterone mimicking, anti-depressant and tumor multidrug resistance reversal activities.[1-9] Most of these medicinal properties have been attributed to its flavonol glycosides (Fig. 1), such as icariin (**9**), baohuoside I (**2a**) and baohuoside II (**1a**). The common characteristic of these active compounds is that they usually have both a rhamnose moiety at the 3-OH position and a prenyl group at the C-8 position.[10] To date, these active rhamnosides are mainly isolated from *Epimedium* plants. However, the rate of renewal of this resource is low and environmentally dependent.[11] The biosynthetic pathway of icariin (**9**) and its derivatives is not yet completely clear.

Previous research has revealed that the prenylation reaction precedes glycosylation after the flavonol skeleton is formed in the biosynthesis of epimedoside A (**8**) in the cell-free extract of *E. diphyllum* cell suspension cultures.[12, 13] A hypothesized biosynthetic pathway for icariin (**9**) in *Epimedium* has been proposed, although the order of rhamnosylation, glucosylation and methylation still remains unknown (Scheme S1, Electronic Supplementary Information, ESI).

Rhamnosyltransferase, the key enzyme involved in the biosynthesis of rhamnoside products, can be characterized and applied to the biosynthesis of bioactive rhamnosides, combined with UDP-rhamnose synthase.[14] However, only two flavonol rhamnosyltransferases, AtUGT78D1 and AtUGT89C1 from *Arabidopsis*, have been identified to date. AtUGT78D1 catalyzes the transfer of rhamnose from UDP-rhamnose to the 3-OH position of quercetin and kaempferol (**3**), whereas AtUGT89C1 converts kaempferol 3-*O*-glucoside to kaempferol 3-*O*-glucoside-7-*O*-rhamnoside (**6**) and recognizes 3-*O*-glycosylated flavonols and UDP-rhamnose as substrates.[14, 15]

Here, we isolate and functionally characterize a rhamnosyltransferase EpPF3RT and a UDP-rhamnose synthase EpRhS from *E. pseudowushanense*. EpPF3RT regiospecifically catalyzes the 3-*O*-rhamnosylation of 8-prenylkaemferol (**1**), anhydroicaritin (**2**) and kaempferol (**3**) combined with EpRhS. Moreover, an engineered *E. coli* strain containing *EpPF3RT* and

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EpRhS was constructed to produce baohuoside II (**1a**) from whole cells by bioconversion.

Results and discussion

Cloning candidate cDNAs from *E. pseudowushanense*

A transcriptome database of *E. pseudowushanense* containing a total of 52,967 unigenes was acquired. The results showed that nine unigenes were both assigned to flavone and flavonol biosynthesis by KEGG (Kyoto Encyclopedia of Genes and Genomes) analysis and with predicted functional annotations as putative UDP-rhamnose: rhamnosyltransferases. After obtaining their full-length sequences, these ORFs (open reading frames) were isolated by nested PCR (polymerase chain reaction). Finally, the ORFs of the nine candidate rhamnosyltransferase cDNAs, which were named EpRTs, were retrieved for further study. A candidate UDP-rhamnose synthase cDNA with a predicted functional annotation as a probable rhamnose biosynthetic enzyme, which was named EpRhS, was also retrieved for further use.

Biosynthesis of UDP-rhamnose using recombinant EpRhS *in vitro*

Since UDP-rhamnose is not commercially available, we used EpRhS, the UDP-rhamnose synthase derived from *E. pseudowushanense*, to synthesize this sugar donor. EpRhS contains a 2022 bp ORF and encodes a 673 amino acid polypeptide with a calculated molecular mass of 75.6 kDa. The multiple alignment of the encoded polypeptides of EpRhS and two reported UDP-rhamnose synthases, RHM2 and OcRhS1,[16, 17] is shown in Fig. S1 (ESI). It shows 84% and 87% sequence identity with RHM2 and OcRhS1, respectively.

The ability of EpRhS to synthesize UDP-rhamnose was identified in combination with AtUGT78D1. Recombinant AtUGT78D1 was expressed in *E. coli*, and the crude extracts containing the recombinant rhamnosyltransferase can transfer a rhamnose moiety to kaempferol (**3**) in the presence of UDP-rhamnose.[14] EpRhS was first incubated with UDP-glucose, NADPH (β-nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt hydrate) and NAD^+ (β-nicotinamide adenine dinucleotide hydrate), and the prepared mixture, after protein removal, was used in the second reaction to detect the rhamnosylation activity of AtUGT78D1. As shown in Fig. S2 (ESI), a product was detected by HPLC-MS with a molecular weight that was 146 amu more than that of kaempferol (**3**), suggesting the rhamnosylation of **3**. This result is in good agreement with previous reports, indicating the ability of EpRhS to synthesize UDP-rhamnose. Thus, UDP-rhamnose was prepared in the same way for use in this study.

Functional characterization of recombinant EpRTs *in vitro*

The coding regions of EpRTs were cloned into the expression vector pET-28a and overexpressed in *E. coli*. For the rhamnosylation activity assay, the crude extracts containing the recombinant EpRTs were incubated with flavonols and flavonol glycosides that may be involved in the biosynthetic

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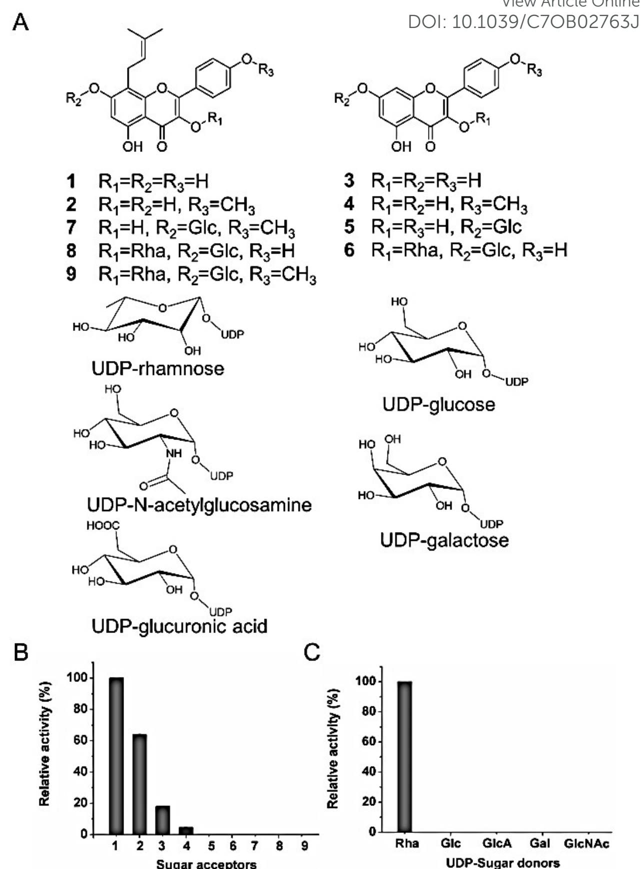

Fig. 1. Substrate specificity of recombinant EpPF3RT. A) Chemical structures of substrates used for the substrate specificity analysis. B) Relative enzymatic activities with flavonols or flavonol glycosides as sugar acceptors and UDP-rhamnose as the sugar donor. C) Relative enzymatic activities with five UDP-sugars (Rha, rhamnose; Glc, glucose; GlcA, glucuronic acid; Gal, galactose; GlcNAc, *N*-acetylglucosamine) as sugar donors and 8-prenylkaemferol (**1**) as the sugar acceptor.

pathway of icariin (**9**) as sugar acceptors in the presence of the UDP-rhamnose prepared above (Fig. 1). Control reactions were performed with crude extracts prepared from bacteria harboring only the empty vector. Subsequently, only EpPF3RT showed rhamnosylation activity. EpPF3RT catalyzed the conversion of the four flavonol aglycones 8-prenylkaempferol (**1**), anhydroicaritin (**2**), kaempferol (**3**) and kaempferide (**4**) to a single rhamnosylated product, respectively (Fig. 2; Fig. S5–Fig. S13, ESI). EpPF3RT showed no activity towards the glycosides that we tested, and it yielded a much higher conversion rate with prenylflavonols like 8-prenylkaempferol (**1**) than with flavonols without a prenyl substitution like kaempferide (**4**). To investigate its sugar donor specificity, UDP-glucose, UDP-glucuronic acid, UDP-galactose and UDP-*N*-acetylglucosamine were also tested as possible sugar donors with **1** as the acceptor. However, EpPF3RT showed no detectable activity toward these sugar donors (Fig. 1).

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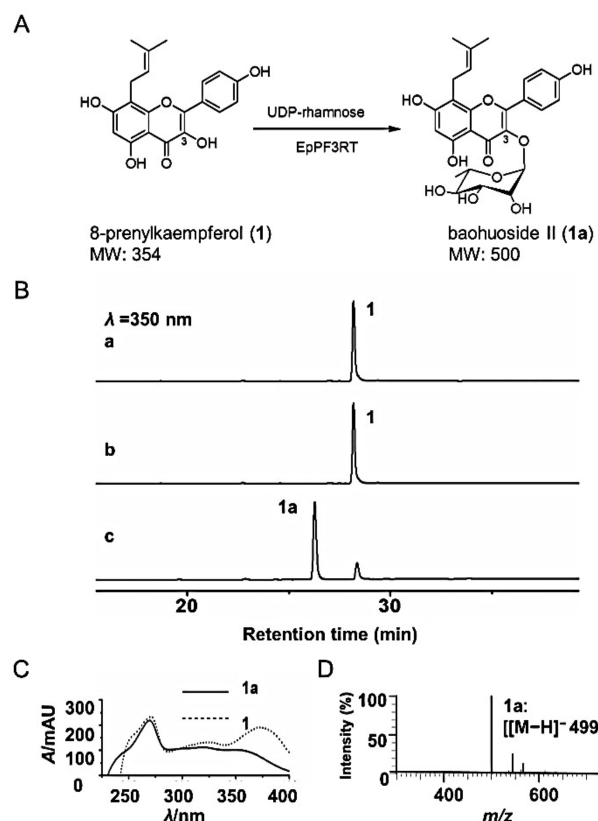

Fig. 2. Functional characterization of recombinant EpPF3RT. A) Enzymatic reaction catalysed by EpPF3RT; B) HPLC/ESI-MS analysis of the enzymatic reaction with 8-prenylkaempferol (**1**). a) Standard of **1**; b) Control group; c) Recombinant EpPF3RT; C) UV spectra of **1** and **1a**. D) MS spectrum of **1a** at negative mode.

To clarify the rhamnosylation property, the enzymatic reactions of the recombinant enzyme with **1**–**3** were scaled up. The products **1a**–**3a** were prepared and further characterized by extensive spectroscopic analysis. In the case of **1a**, for example, one product with higher polarity was detected in the reaction mixture by HPLC-UV/ESI (Fig. 2). Selected ion monitoring for *m/z* 499 $[M-H]^-$ suggested that the product was a mono-rhamnosylated product with a molecular weight that was 146 amu higher than that of **1** (Fig. 2B, 2D). No activity was observed in the control group. The shifting of the maximum UV absorption in band I of **1** (370 nm) to a shorter wavelength (350 nm) in **1a** suggests 3-*O*-rhamnosylation (Fig. 2C). In its ^{1}H NMR spectrum, the appearances of a proton at δ_H 5.26 (1H, d, J = 1.6 Hz, H-1''), a methyl at δ 0.78 (3H, d, J = 6.0 Hz, H-6'') and four protons at δ_H 3.10–3.98 indicate the introduction of an α type rhamnose moiety. The observation of a downfield shift for C-2 and an upfield shift for C-3 in the ^{13}C NMR spectrum compared to that of **1** confirmed this deduction (Table S4).[18] All of the data are in good agreement with the previously reported data for baohuoside II (**1a**).[19] Thus, the enzymatic product **1a** was unequivocally identified as

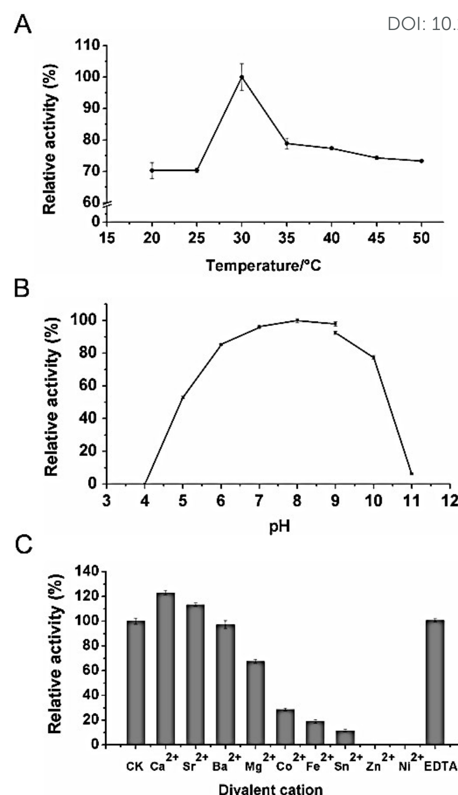

Fig. 3. Effects of temperature (A), pH (B) and various divalent metal ions (C) on enzyme activity of EpPF3RT. UDP-rhamnose was used as sugar donor and 8-prenylkaemferol (**1**) was used as aglycon acceptor. The values are presented as the means, and the error bars show the SD (n = 3).

Table 1 Catalytic activity of EpPF3RT towards different substrates.

	EpPF3RT ***v*** (nmol min^{-1} mg^{-1})
Sugar acceptor [a]	
1	2.86
2	2.52
3	0.10
4	0.07
Sugar donor [b]	
UDP-rhamnose	2.86

[a]The reactions were performed with UDP-rhamnose as the sugar donor.
[b]The reactions were performed with 8-prenylkaemferol (**1**) as the sugar acceptor.

baohuoside II (**1a**). EpPF3RT was identified as a flavonol 3-*O*-rhamnosyltransferase that regiospecifically transfers a

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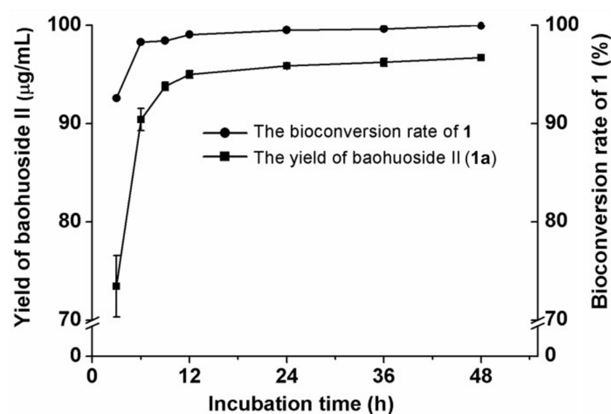

Fig. 4. Time course for the biotransformation of 8-prenykaempferol (**1**) using strain B-PKR. The values are presented as the means, and the error bars show the SD (n = 3).

rhamnose moiety to prenyl flavonol at the 3-OH position. EpPF3RT shows an ORF of 1422 bp encoding a protein of 473 amino acids with a calculated molecular mass of 52.1 kDa. A multiple sequence alignment of the encoded polypeptides of EpPF3RT and the reported flavonol rhamnotransferases AtUGT78D1 and AtUGT89C1 is shown in Fig. S3 (ESI). Highly conserved sequences of the GTs, PSPG (plant secondary product glycosyltransferase) motif, were observed in these polypeptides. Moreover, EpPF3RT shared identities of 20% at the amino acid level with AtUGT78D1 and 17% with AtUGT89C1. A phylogenetic tree was constructed and reveals that EpPF3RT and other plant GTs are grouped into a single clade with the highest identity (41%) with CsF7G6"RT from *Citrus sinensis* (Fig. S4, ESI).

Biochemical properties of recombinant EpPF3RT

As indicated in Fig. 3, EpPF3RT showed a broad optimum in a temperature range of 20–50 °C, and its maximum activity was observed at 30 °C. Analysis of the enzyme activity between pH 4.0 and 11.0 showed that the optimal pH value was between 6.0 and 10.0 and that the maximum activity was observed at pH 8.0. The catalytic activity decreased rapidly at pH values beyond this range. The effect of divalent cations on the activity showed that this enzyme was independent of metal ions, including Mg^{2+}. Ca^{2+} and Sr^{2+} can improve the activity, while the other divalent cations decreased the activity. The rhamnosylation activity was observed to decrease in the order of $Ca^{2+}>Sr^{2+}>Ba^{2+}>Mg^{2+}>Co^{2+}>Fe^{2+}>Sn^{2+}$. Since UDP-rhamnose is difficult to purify from the UDP-rhamnose-synthesized mixture, we examined rhamnosylation velocities instead of kinetic analyses using the same UDP-rhamnose preparation mixture.[20] The rhamnosylation velocity of **1** was the highest with a reaction rate of 2.86 nmol min^{-1} mg^{-1} when compared with the other three substrates, as shown in Table 1, indicating that 8-prenylkaempferol (**1**) may be the native substrate of EpPF3RT.

Bioconversion with engineered *E. coli*

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Whole cell biocatalysis offers some unique advantages. Multi-step reactions in a single strain with cofactor regeneration, high catalytic efficiency and mild conditions are all very attractive for commercial applications.[21] In addition, whole cell biocatalysis can remove the need for expensive precursors like UDP-glucose and the isolation of multiple recombinant proteins. Therefore, whole cell bioprocesses hold promise as commercially viable routes to produce active compounds.
For the bioconversion of exogenously supplemented 8-prenylkaempferol (**1**) using engineered *E. coli*, the pCDFDuet vector carrying *EpPF3RT* and *EpRhS* was inducted into *E. coli* BL21 (DE3), and the transformant was designated B-PKR. *E. coli* BL21 (DE3) containing the empty pCDFDuet vector did not produce any detectable products, whereas the B-PKR strain produced a single product, which was determined to be baohuoside II (**1a**). As a result, 96.7 μg mL^{-1} of baohuoside II (**1a**) with a conversion rate of **100%** was produced by this biosystem after 48 h of incubation with 8-prenylkaempferol (**1**) (Fig. 4). This module could be used as a part of further metabolic engineering. When supplementing with anhydroicaritin (**2**), kaempferol (**3**) or kaempferide (**4**), low activity with a conversion rate of less than 10% conversion was obtained in 48 h. Furthermore, in order to produce bioactive rhamnosides using microbial systems with cheaper precursors, extra enzymes would need to be engineered into the microbial systems to mimic the natural biosynthetic pathway. That is, if we combined a prenyltransferase, a glycosyltransferase, and a methyltransferase or even the enzymes responsible for the formation of the flavonol skeleton, a more economical, effective and environmentally friendly microbial factory could be built to supply the related active compounds.

Experimental section

General methods

UDP-glucose, NADPH and NAD^+ were purchased from Sigma-Aldrich (St. Louis, USA), and the substrates in Fig. 1 (**1**–**9**) tested for enzymatic reactions were purchased from Nantong Feiyu Biological Technology Co., Ltd. (Nantong, China). The analyses of the substrate specificity and the determinations of the conversion rates were performed using an Agilent 1200 series HPLC system (Agilent Technologies, Germany) coupled with an LCQ Fleet ion trap mass spectrometer (Thermo Electron Corp., USA) equipped with an electrospray ionization (ESI) source. Compounds were characterized by ^{1}H NMR at 400 or 600 MHz and ^{13}C NMR at 100 or 150 MHz using a Mercury-400 spectrometer and a Bruker AVIIIHD-600 spectrometer. The chemical shifts (δ) were referenced to internal solvent resonances and are given in parts per million (ppm). Coupling constants (J) are given in hertz (Hz).

Plant materials

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Fresh leaves of *E. pseudowushanense* were collected and immediately frozen with liquid nitrogen and then stored at −80 °C until use. *E. pseudowushanense* was planted in Guizhou Province, China, and identified by one of the authors, Prof. Guo.

Functional annotation of unigenes from *E. pseudowushanense* transcriptome data

Transcriptome sequencing of *E. pseudowushanense* was performed by the Beijing Genomics Institute. In brief, a cDNA library derived from mRNA of *E. pseudowushanense* was sequenced using Illumina HiSeq™ 2000. A large number of short reads was hence generated from the sequencing. These resulting raw reads were then combined by Trinity, an assembling program, to form longer fragments that could not be extended at either end, which were defined as unigenes. The unigenes were then annotated by blastx against GenBank databases like NR, Swiss-Prot, KEGG and COG (e-value < 0.00001). The unigene sequences were firstly aligned to protein databases like NR, Swiss-Prot, KEGG and COG (e-value<0.00001) by blastx, and to the nucleotide database NT (e-value<0.00001) by blastn, retrieving the proteins with the highest sequence similarity to the given unigenes along with their protein functional annotations. Nine candidate unigenes, which were annotated to be UDP-rhamnosyltransferases and were responsible for the flavone and flavonol biosynthesis pathway based on KEGG pathway analysis, were retrieved for further study. Another candidate unigene, *EpRhS*, was also retrieved for further use. It was presumably similar to RHM2, a multidomain protein that converts UDP-glucose to UDP-rhamnose in *Arabidopsis*.[16]

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Isolation, expression and purification of the EpPF3RT

Total RNA of the leaves of *E. pseudowushanense* was prepared by using the E.Z.N.A.TM Plant RNA Kit (Omega Bio-Tek, USA) and reverse-transcribed to cDNA with SmartScribe reverse transcriptase (Clontech, USA) following the manufacturer's instructions. The unigenes were analyzed using the online bioinformatics tools from NCBI. The open reading frame finding was performed using the on-line program (https://www.ncbi.nlm.nih.gov/orffinder/). Six of the nine candidate rhamnosyltransferase unigenes were provided with sequences containing the predicted ORFs, which can be isolated from *E. pseudowushanense* cDNA directly by nested PCR using KOD Plus Neo polymerase and gene-specific primers. The missing sequences of the three other tentative partial cDNAs, however, were obtained by RACE (rapid amplification of cDNA end). After obtaining the full-length sequences, the ORFs of them were also isolated by nested PCR. Finally, the ORFs of the nine candidate rhamnosyltransferase cDNAs, which were named EpRTs, were retrieved for further study (Table S3, ESI).

The PCR products were purified and cloned into the pET-28a vector using an EZfusion Enzyme (Shanghai Generay Biotech Co., Ltd, China). After verification of the sequences, the recombinant plasmid and empty pET-28a were transformed into *Transetta* (DE3) *E. coli* (TransGen Biotech, China) for heterologous expression. The described *E. coli* strains were cultivated in 1-L shake flasks at 37 °C and 200 rpm using 200 mL LB (Luria-Bertani) medium containing 50 μg mL^{-1} kanamycin and 34 μg mL^{-1} chloromycetin until they reached an OD_{600} (optical density at 600 nm) of 0.4–0.6. The recombinant N-terminal His_6-EpRTs expression was achieved by induction with 0.1 mM IPTG (isopropyl-β-D-thiogalactoside) for 16 h at 18 °C.

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The cells were harvested by centrifugation at 10,000 g for 5 min at 4 °C. The cells were resuspended in 20 mL Tris-HCl buffer (50 mM Tris-HCl buffer, 50 mM NaCl, 1 mM DTT, 5% glycerol, pH 7.4) and disrupted by sonication in an ice bath. The cell debris was removed by centrifugation at 10,000 g and 4 °C for 60 min, and the supernatant was collected as crude extracts. For purification, the harvested cells were resuspended in 20 mL binding buffer (20 mM phosphate buffer, 0.5 M NaCl, 20 mM imidazole, pH 7.4) containing 1 mM PMSF (phenylmethylsulfonyl fluoride). The cells were also disrupted by sonication in an ice bath, and the cell debris was removed by centrifugation at 10,000 g and 4 °C for 60 min. The soluble fraction was passed through a 0.45-μm syringe filter unit, and the cleared supernatant was immediately applied to a 1-mL column of Ni-NTA resin (GE, USA) that was pre-equilibrated with binding buffer. The resin was subsequently eluted with 5 mL (5 CVs) of washing buffer (20 mM phosphate buffer, 0.5 M NaCl, 50 mM imidazole, pH 7.4). The elution was carried out with 5 mL (5 CVs) of different elution buffers (20 mM phosphate buffer, 0.5 M NaCl, 75–250 mM imidazole, pH 7.4). The protein purification was performed with a flow rate of 1 mL min^{-1} and a temperature of 4 °C. The proteins were concentrated and buffer exchanged to a desalting buffer (50 mM Tris-HCl buffer, 50 mM NaCl, 1mM DTT, 5% glycerol, pH 7.4) using an Amicon Ultra-30K centrifugal concentrator (Millipore). The protein purity was confirmed by SDS-PAGE (Fig. S14), and the protein concentration for all of the studies was determined using the Protein Quantitative Kit (TransGen Biotech, China). The final protein was flash-frozen in liquid nitrogen and stored at −80 °C. The approximate protein yield for EpPF3RT (52.1 kDa) was 9.2 mg L^{-1}.

Activity assays *in vitro*

The UDP-rhamnose biosynthetic mixture containing 0.2 mM UDP-glucose, NADPH, NAD^+ and EpRhS was incubated at 30 °C for up to 12 h. The mixture was boiled at 100 °C for up to 10 min and centrifuged at 15,000 g for 20 min to remove the protein. The supernatant was then incubated with 0.1 mM aglycon, 50 mM Tris-HCl (pH 7.4) and crude extracts of strains containing the recombinant EpRTs in a final volume of 200 μL. Control experiments were performed with the crude extracts of strains containing the empty vector. The activity assays, initiated by the addition of the enzymes, were performed at 30 °C for up to 6 h and terminated by the addition of 400 μL of ice cold MeOH. Subsequently, the samples were centrifuged at 15,000 g for 20 min to collect the supernatant and then analyzed by HPLC-UV/ESIMS, as described in the general

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methods. For quantification, three parallel assays were routinely carried out. The HPLC peak areas were integrated with Agilent Workstation Software, and the total percent conversion was calculated as a percentage of the total peak area of the substrate and the product. The HPLC analyses were performed on a Shiseido capcellpak C_{18} MG III column (250 mm × 4.6 mm I.D., 5 μm, Shiseido Co., Ltd., Tokyo, Japan) at a flow rate of 1 mL min^{-1}, with a column temperature maintained at 30 °C. The mobile phase was a gradient elution of solvent A (0.1% formic acid aqueous solution) and solvent B (methanol). Gradient programs were used for the analyses of the reactions (Table S1, ESI). The UDP-rhamnose synthesis activity of EpRhS was identified in combination with AtUGT78D1 by detecting the rhamnosylation product. The protein expression, purification and quantitation were conducted using the same procedures as mentioned above.

Preparative scale reactions

A total of 11.0 μmol of aglycon **1**, 9.0 μmol of aglycon **2**, and 20.0 μmol of aglycon **3** were dissolved in 200 μL DMSO and diluted with a buffer solution (50 mM Tris-HCl, pH 7.4, 25 mL total volume), respectively. UDP-Glucose, NADPH and NAD^+ were added at twice the molar amount as the aglycons, along with 25 mL of the purified enzymes EpPF3RT and EpRhS extracted from 3 g of wet induced *E. coli* cells T-RT and T-RhS, respectively (Table S3). The reactions were performed at 30 °C for up to 12 h, followed by extraction with ethyl acetate (5 × 100 mL). The organic phase was evaporated to dryness under reduced pressure, and the residue was dissolved in 1.5 mL of methanol and purified by reverse-phase semi-preparative HPLC. The obtained products were weighed, and yields of 96% (10.6 μmol), 87% (7.8 μmol) and 41% (8.1 μmol) were calculated, respectively. The structures were identified by MS, ^{1}H NMR and ^{13}C NMR.

^{1}H and ^{13}C NMR data of prepared glycosylated products

Baohuoside II (**1a**). ^{1}H NMR (DMSO-d_6, 600 MHz): δ_H 0.78 (3H, d, J = 6.0 Hz H-6''), 1.61 (3H, s, H-14), 1.67 (3H, s, H-15), 3.48 (2H, m, H-11), 5.14 (1H, t, J = 7.2 Hz, H-12), 5.26 (1H, d, J = 1.6, Hz H-1''), 6.30 (1H, s, H-6), 6.90 (2H, d, J = 8.8 Hz, H-3', H-5'), 7.74 (2H, d, J = 8.8 Hz, H-2', H-6'), 10.84 (1H, br. s, 7-OH), 12.53 (1H, s, 5-OH), 3.10–3.98 (4H, protons in rhamnose). ^{13}C NMR (DMSO-d_6, 125 MHz): δ_C 177.9 (C-4), 161.6 (C-5), 161.3 (C-7), 158.8 (C-4'), 156.8 (C-2), 153.8 (C-9), 134.4 (C-3), 131.0 (C-13),130.4 (C-2', 6'), 122.4 (C-1'), 122.3 (C-12), 114.1 (C-3', 5'), 105.9 (C-8), 104.2 (C-10), 101.8 (C-1''), 98.3 (C-6), 70.3 (C-4''), 70.1 (C-2''), 70.6 (C-3''), 71.1 (C-5''), 17.5 (C-6''), 25.4 (C-14), 21.2 (C-11), 17.8 (C-15), 17.5 (C-6''). ESI-MS: m/z = 499 $[M–H]^-$. All of these data are in good agreement with the previously reported data for baohuoside II (**1a**).[19]

Baohuoside I (**2a**). ^{1}H NMR (DMSO-d_6, 600 MHz): δ_H 0.78 (3H, d, J = 6.0 Hz, H-6''), 1.61 (3H, s, H-14), 1.67 (3H, s, H-15), 3.48 (2H, m, H-11), 5.15 (1H, t, J = 6.6 Hz, H-12), 5.27 (1H, d, J = 1.6 Hz, H-1''), 6.29 (1H, s, H-6), 7.11 (2H, d, J = 8.9 Hz, H-3', H-5'), 7.85 (2H, d, J = 8.8 Hz, H-2', H-6'), 10.84 (1H, br. s, 7-OH), 12.53 (1H, s, 5-OH), 3.03–3.98 (4H, protons in rhamnose). ^{13}C NMR (DMSO-d_6, 125 MHz): δ_C 177.9 (C-4), 162.2 (C-7), 161.2 (C-4'), 158.8 (C-5), 156.6 (C-2), 153.8 (C-9), 134.4 (C-3), 130.9 (C-13), 130.4 (C-2', 6'), 122.5 (C-1'), 122.4 (C-12), 114.0 (C-3', 5'), 106.0 (C-8), 103.9 (C-10), 101.9 (C-1''), 98.5 (C-6), 70.3 (C-4''), 70.1 (C-2''), 70.6 (C-3''), 71.1 (C-5''), 55.5 (OCH_3), 25.4 (C-14), 21.2 (C-11), 17.8 (C-15), 17.5 (C-6''). ESI-MS: m/z 513 $[M–H]^-$. All of these data are in good agreement with the previously reported data for baohuoside I (**2a**).[22]

Afzelin (**3a**). ^{1}H NMR (Methanol-d_4, 400 MHz): δ_H 7.77 (2H, d, J = 6.80 Hz, H-2', 6'), 6.93 (2H, d, J = 6.76 Hz, H-3', 5'), 6.38 (1H, d, J = 2.08 Hz, H-8), 6.21 (2H, d, J = 2.12 Hz, H-6), 5.38 (1H, d, J = 1.68 Hz, H-1''), 4.21 (1H, dd, J = 1.76, 3.40 Hz, H-2''), 3.71 (H, m, H-3''), 0.92 (3H, d, J = 5.6 Hz, H-6''). ^{13}C NMR (Methanol-d_4, 100 MHz): δ_C 179.6 (C-4), 165.9 (C-7), 163.3 (C-5), 161.6 (C-4'), 159.3 (C-9), 158.6 (C-2), 136.2 (C-3), 131.9 (C-2', 6'), 122.6 (C-1'), 116.5 (C-3', 5'), 105.9 (C-10), 103.5 (C-1''), 99.8 (C-6), 94.7 (C-8), 73.2 (C-4''), 71.9 (C-2''), 72.0 (C-3''), 72.1 (C-5''), 17.7 (C-6''). ESI-MS: m/z 431 $[M–H]^-$. All of these data are in good agreement with the previously reported data for afzelin (**3a**).[23]

Biochemical properties of recombinant EpPF3RT

To assay for the optimal reaction temperature, the reaction mixtures were incubated at different temperatures (20–50 °C). To study the optimal pH, the enzymatic reaction was performed in various reaction buffers with pH values in the range of 4.0–7.0 (citric acid-sodium citrate buffer), 7.0–9.0 (Tris-HCl buffer) and 9.0–11.0 (Na_2CO_3-$NaHCO_3$ buffer). To test the effects of divalent metal ions on EpPF3RT activity, $BaCl_2$, $CaCl_2$, $CoCl_2$, $FeCl_2$, $MgCl_2$, $SnCl_2$, $SrCl_2$, $NiCl_2$, and $ZnCl_2$ were used individually at final concentrations of 5 mM. The assays were performed with UDP-rhamnose as the donor and 8-prenylkaempferol (**1**) as the acceptor. To examine the rhamnosylation velocity, the enzymatic reaction was performed with 50 μg of purified EpPF3RT at the optimal temperature and pH and was incubated for up to 30 min. The rhamnosylation activity was analyzed as mentioned above.

Bioconversion with engineered *E. coli*

EpPF3RT and *EpRhS* were subcloned into pCDFDuet (Novagen). The resulting construct was named pC-PF3RT-RhS (Table S3, ESI). The construct was transformed into *E. coli* strain BL21 (DE3), and the resulting transformant was named B-PKR. The B-PKR strain was used to biotransform 8-prenylkaempferol (**1**), anhydroicaritin (**2**), kaempferol (**3**) and kaempferide (**4**). One-hundredth of the volume of the overnight-induced culture was inoculated in fresh LB media, and the culture was grown until the OD_{600} reached 0.8. The enzymes were induced by adding 1 mM IPTG to the culture, and the culture was incubated at 20 °C for 20 h. The cells were harvested by centrifugation and resuspended to obtain a cell concentration corresponding to an OD_{600} of 3 in 1 mL M9 medium (pH 7.0, Na_2HPO_4 6.8 g L^{-1}, KH_2PO_4 3 g L^{-1}, NaCl 0.5 g L^{-1}, NH_4Cl 1 g L^{-1}, $MgSO_4$ 0.24 g L^{-1}, $CaCl_2$ 0.01 g L^{-1}) containing 2% glucose.[24] After the four substrates (200 μM) were added to the cells, the reaction mixture was incubated at 30 °C for 72 h. The reaction mixture

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(40 μL) was collected at 3, 6, 9, 12, 24, 36, and 48 h. The reaction was terminated by the addition of 100 μL of ice cold MeOH and centrifuged. The supernatant was used for HPLC analysis. HPLC analyses were performed using the conditions mentioned in Table S2. Three independent experiments were performed, and the quantitative analysis was further defined by the external standard method. The HPLC peak areas were integrated using Agilent Workstation Software. The linear regression models between the peak area and the quantity of standards were established for quantitative analysis of the bioconversion. The models and the regression equations are given in Fig. S15 (ESI).

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Accession numbers

The nucleotide sequences of EpPF3RT and EpRhS are available in the GenBank database under accession numbers MG264429 and MG264430, respectively.

Conclusions

In summary, a regiospecific rhamnosyltransferase EpPF3RT from *E. pseudowushanense* that catalyzes the 3-*O*-rhamnosylation of 8-prenylkaemferol (**1**), anhydroicaritin (**2**) and kaempferol (**3**) was characterized. It possesses strict substrate specificity and regiospecificity and can be used as an environmentally friendly and efficient biocatalyst for the preparation of bioactive flavonol rhamnosides. Additionally, the flavonol 3-*O*-rhamnoside baohuoside II (**1a**) was efficiently synthesized in *E. coli* after the introduction of a vector combining *EpPF3RT* with *EpRhS*, with 8-prenylkaempferol (**1**) supplementation of the culture. Furthermore, bioactive compounds such as icariin (**9**), baohuoside I (**2a**), and baohuoside II (**1a**) may be synthesized from simpler molecules by further design of the microbial systems, and the rhamnosylation module in this study may serve as a component of these systems.

Conflicts of interest

The authors declare no competing financial interest.

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