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NOTE

Biotransformation of quercetin to quercetin 3-*O*-gentiobioside using engineered *Escherichia coli*

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Abstract Various flavonoid *O*-diglycosides are found in nature but the biological activities of only a few compounds have been explored due to difficulty in obtaining samples. In order to circumvent the need for extraction and purification of the natural compounds from plants, we used engineered *Escherichia coli* strain that harbors two uridine diphosphate-dependent glycosyltransferases (UGTs; *BcGT1* and *CaUGT*) and two nucleotide sugar biosynthetic genes (*pgm* and *galU*). Using this strain, we synthesized quercetin 3-*O*-gentiobioside. After optimization of induction temperature, cell density, and reaction temperature, approximately 46.2 mg/L quercetin 3-*O*-gentiobioside was synthesized from quercetin with 49 % conversion efficiency.

Keywords Glycosyltransferase · Nucleotide sugar biosynthesis · Quercetin 3-*O*-gentiobioside

Introduction

One of phytochemicals, flavonoid, has roles in pigmentation, UV-B protection, auxin transport, and signaling for interaction with microorganisms in plants (Iwashina 2003). Flavonoids exist not only as aglycones but as glycones. Glycosylation reactions occur after synthesis of flavonoids by uridine diphosphate-dependent glycosyltransferases (UGTs). Sugars are attached to the flavonoids, which increases the diversity of flavonoids (Vogt and Jones 2000).

Flavonoid *O*-monoglycosides are the most prevalent forms. Flavonoid *O*-biglycosides and flavonoid *O*-diglycosides are found. For example, quercetin 3-*O*-glucose 7-*O*-rhamnose and kaempferol 3-*O*-glucose 7-*O*-rhamnose were found in *Arabidopsis thaliana* (Jones et al. 2003). Neohesperidin (hesperetin 7-*O*-glucose (1 → 2) rhamnose), naringin (naringenin 7-*O*-glucose (1 → 2) rhamnose), hesperidin (hesperetin 7-*O*-rhamnose (1 → 6) glucose), and narirutin (naringenin 7-*O*-rhamnose (1 → 6) glucose) were found in citrus (Peterson et al. 2006a, b; Rousseff et al. 1987). Thus, flavonoid *O*-diglycosides have a role in determining the taste of fruits (Frydman et al. 2004). Quercetin 3-*O*-gentiobioside was found in *Catharanthus roseus* and the corresponding gene was cloned (Masada et al. 2009). Although various flavonoid *O*-diglycosides were found, the biological activities of only a few compounds such as rutin (quercetin 3-*O*-glucosyl (1 → 6) rhamnoside) have been explored due to the difficulty in obtaining samples (La Casa et al. 2000; Guardia et al. 2001; Sheu et al. 2004). In order to circumvent the need for extraction and purification of the natural compounds from plants, microbial production of natural products is an emerging field (Jeandet et al. 2013; Zhou et al. 2015). We reported here the biological synthesis of quercetin 3-*O*-gentiobioside (glucose (1 → 2) glucose) using engineered *E. coli*.

Materials and methods

Constructs

In order to clone *CaUGT* from *C. roseus* (GenBank accession number AB443870), cDNA was synthesized

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using the whole plant as described previously (Kim et al. 2012) and polymerase chain reaction (PCR) was carried out. The primers were 5′-AAgtcgacATGGCCACAGAACAACAACA-3′ (the lower case letters indicate a SalI site) and 5′-AAgcggccgcTCACACACACAGCTGCTTCA-3′ (the lower case letters indicate a NotI site). The gene *pgm* (phosphoglucomutase; Gene ID 945271) and *galU* (uridine triphosphate alpha-D-glucose-1-phosphate uridylyltransferase; Gene ID 945730) from *E. coli* were cloned by PCR. The primers for *galU* were 5′-AAgaattcATGGCTGCCATTAATACGAAAG-3′ (the lower case letters indicate an *Eco*RI site.) and 5′-AAgcggccgcTTACTTCTTAATGCCCATCTCTTC-3′ (the lower case letters indicate a *Not*I site.). The primers for pgm were 5′-ATGcatatgGCAATCCACAATCGTGCAGGC-3′ (the lower case letters indicate a *Nde*I site.) and 5′-CATctcgagGTTACGCGTTTTTCAGAACTTC-3′ (the lower case letters indicate an *Xho*I site.). All PCR products were sequenced. *CaUGT* was cloned into the *Sal*I/*Not*I sites of pGEX5X-3 (GE Healthcare Life Sciences, Pittsburgh, PA, USA; pG-CaUGT). *pgm* and *galU* were cloned into pCDFDuet-1 vector (Novagen; pC-pgm-galU). The strain B-BcGT1 was constructed previously (An et al. 2016).

Synthesis of quercetin 3-*O*-gentiobioside using *E. coli*

Escherichia coli strain B-BcGT1 was transformed with pG-CaUGT and pCDF (strain B-QGB1) or pG-CaUGT and pC-pgm-galU (strain B-QGB2). The production of quercetin 3-*O*-gentiobioside in the *E. coli* transformant was compared. Each transformant was grown into Luria–Bertani (LB) medium containing 50 μg/mL spectinomycin and 50 μg/mL of ampicillin. Cells were inoculated into a fresh medium and grown until the optical density at 600 nm (OD_{600}) reached 1.0 at which point the proteins were induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) to the culture at a final concentration of 1 mM. Culture was incubated for 24 h at 18 °C. After collection of cells, the cell concentration was adjusted to an OD_{600} of 1.0 with 2 mL of M9 medium supplemented with 2 % glucose, 50 μg/mL spectinomycin, and 50 μg/mL ampicillin. Quercetin [50 μM; dissolved in dimethyl sulfoxide (DMSO)] was also added, and the culture was incubated at 30 °C with shaking for 7 h. The culture supernatant was boiled for 3 min and centrifuged for 15 min. The supernatant was analyzed by HPLC (Yoon et al. 2012).

To measure the production of quercetin 3-*O*-gentiobioside, *E. coli* strain B-QGB2 was grown as described above. The cells were resuspended in M9 with an optical density of $OD_{600} = 3$. Quercetin (50 μM) was added to the culture at 0, 10, and 18 h (for a final concentration of 150 μM). Samples were periodically harvested and the supernatant was analyzed by HPLC.

The reaction product was purified as described in An et al. (2016) and its structure was determined using NMR spectroscopy (Yoon et al. 2012). ^{1}H NMR (MeOD-d_4) δ ppm 3.01 (m, H-5‴), 3.06 (dd, $J = 7.7$, 9.3 Hz, H-2‴), 3.17 (t, $J = 8.9$ Hz, H-3‴), 3.19 (t, $J = 9.0$ Hz, H-4‴), 3.38 (m, *H-4″*), 3.39 (m, *H-5″*), 3.42 (m, *H-3″*), 3.50 (dd, $J = 7.6$, 9.1 Hz, H-2″), 3.56 (dd, $J = 5.4$, 12.1 Hz, H-6‴), 3.67 (dd, $J = 5.5$, 12.0 Hz, H-6″), 3.75 (dd, $J = 2.1$, 12.1 Hz, H-6‴), 3.98 (dd, $J = 1.6$, 12.0 Hz, H-6″), 4.15 (d, $J = 7.6$ Hz, H-1‴), 5.20 (d, $J = 7.6$ Hz, H-1″), 6.18 (d, $J = 2.1$ Hz, H-6), 6.38 (d, $J = 2.1$ Hz, H-8), 6.87 (d, $J = 8.4$ Hz, H-5′), 7.66 (dd, $J = 2.2$, 8.4 Hz, H-6′), 7.70 (d, $J = 2.2$ Hz, H-2′); ^{13}C NMR δ ppm 62.9 (C-6‴), 69.9 (C-6″), 71.6 (C-4″), 71.7 (C-4‴), 75.4 (C-2‴), 76.1 (C-2″), 77.9 (C-5‴), 78.0 (C-3‴), 78.2 (C-5″), 78.3 (C-3″), 95.2 (C-8), 100.4 (C-6), 104.3 (C-1″), 104.9 (C-1‴), 116.3 (C-5′), 117.7 (C-2′), 123.8 (C-6′).

Results and discussion

Quercetin 3-*O*-gentiobioside has two molecules of glucose. It is synthesized from quercetin by two sequential glucosylation catalyzed by two UGTs (Fig. 1). The first reaction is the synthesis of quercetin 3-*O*-glucoside from quercetin, which is mediated by BcGT1 (Ko et al. 2006). The second reaction, which is catalyzed by CaUGT, is the synthesis of quercetin 3-*O*-gentiobioside from the first reaction product. *BcGT1* is needed to be retained as a low copy number as possible because the conversion rate of quercetin into quercetin 3-*O*-glucoside is fast, which resulted in the accumulation of quercetin 3-*O*-glucoside without conversion of it into quercetin 3-*O*-gentiobioside. Therefore, we used *E. coli* strain B-BcGT1, in which BcGT1 was integrated into its chromosome. B-BcGT1 harboring pG-CaUGT was used for the production of quercetin 3-*O*-gentiobioside. Figure 2 shows a new peak with a molecular mass of 625.8 Da, which was the predicted molecular mass of quercetin 3-*O*-gentiobioside. In addition, we determined the structure of this product using NMR. All 19 ^{1}H signals were observed. Among the 19 signals, five signals were shown in the aromatic region, meaning that these signals are coming from quercetin. Two peaks at 5.20 and 4.15 ppm indicate that two glucose molecules are present. The remaining ^{1}H peaks were assigned using the COSY spectrum. The connection between the two glucose molecules was confirmed using NOESY experiments. Because a cross peak between 3.67 ppm (H-6 of 3-Glc) and 4.15 ppm (H-1 of 6″-Glc) was observed, the two glucose molecules were linked with 1 → 6 glucoside bond. In addition, both H-1 signals of glucose were connected with H-3 and H-5 in the NOESY spectrum indicating α-glucose. Therefore, we concluded that the structure of the reaction product is quercetin 3-*O*-gentiobioside.

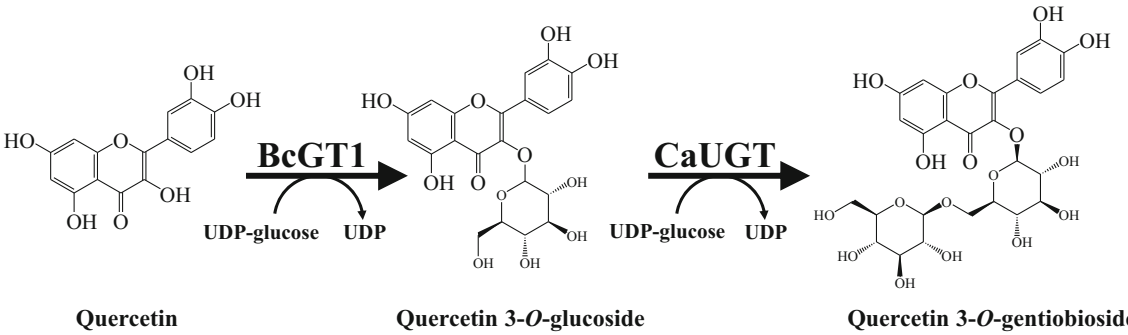

Fig. 1 Synthesis of quercetin 3-*O*-gentiobioside using *Escherichia coli* harboring two glycosyltransferases. *BcGT1*, which was integrated into the *E. coli* chromosome, converts quercetin to quercetin 3-*O*-glucoside. CaUGT converts quercetin 3-*O*-glucoside to quercetin 3-*O*-gentiobioside

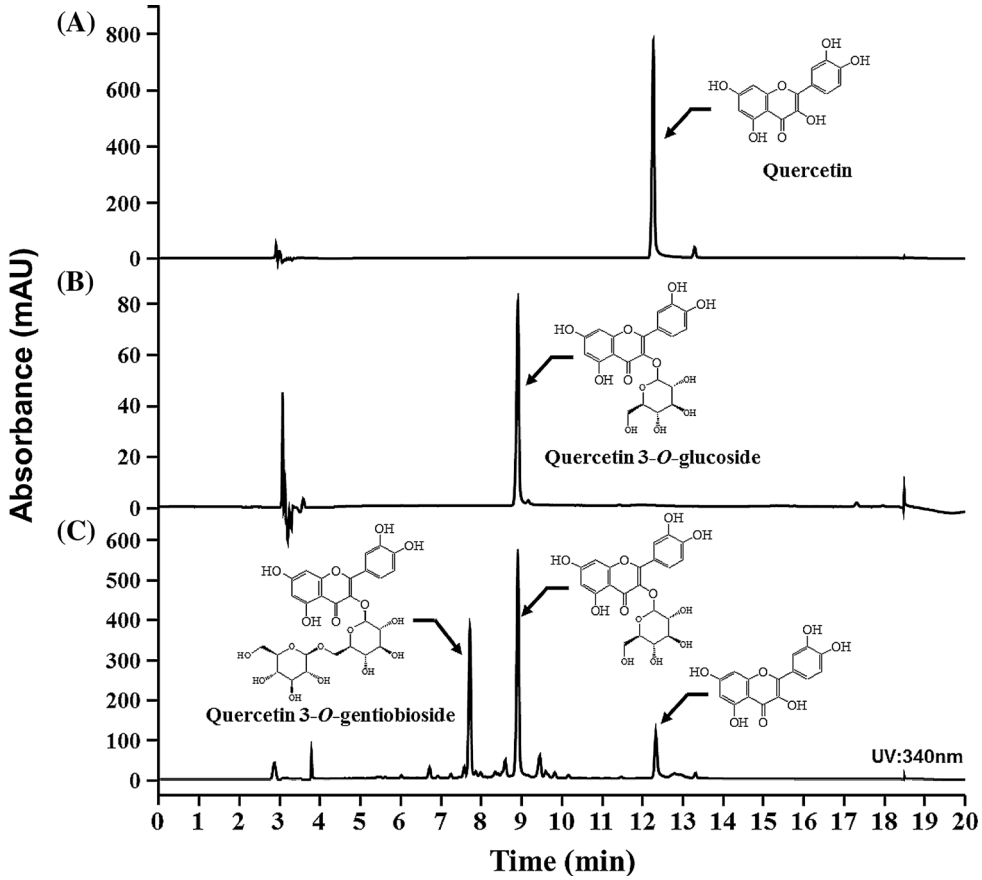

Fig. 2 Analysis of the incubation product produced by *E. coli* strain B-QGB1. (**A**) Quercetin standard, (**B**) quercetin 3-*O*-glucoside standard, and (**C**) reaction product from *E. coli* strain B-QGB1

In order to synthesize quercetin 3-*O*-gentiobioside, UDP-glucose was used as a glucose donor. UDP-glucose is synthesized from UTP and glucose 1-phosphate by ugd in *E. coli*. Glucose 1-phosphate is synthesized from glucose 6-phosphate by the action of pgm. It is known that the overexpression of *pgm* and *galU* increased UDP-glucose in *E. coli* (Mao et al. 2006). We overexpressed two genes, *pgm* and *galU*, in *E. coli* harboring pG-CaUGT. *E. coli* transformants supplemented with 50 μM quercetin were grown at 30 °C for 7 h. *E. coli* harboring pG-CaUGT and pC-pgm-galU (B-QGB2; 6.2 mg/L) produced more quercetin 3-*O*-gentiobioside than *E. coli* harboring only pG-CaUGT (B-QGB1; 4.2 mg/L). This suggested that the pool of UDP-glucose had an influence on the production of quercetin 3-*O*-gentiobioside.

We tested the effect of the induction temperature, initial cell density, and reaction temperature on the production of quercetin 3-*O*-gentiobioside using B-QGB2. Two different temperatures (18 and 30 °C) were tested to determine the better induction temperature. After induction at the two

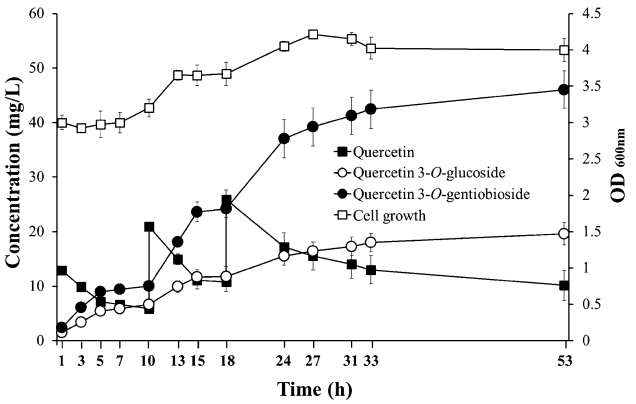

Fig. 3 Production of quercetin 3-*O*-gentiobioside using *E. coli* strain B-QGB2. After induction at 18 °C for 24 h, the cell density was adjusted to an OD_{600} of 3.0 with M9 containing 1 % yeast extract, 2 % glucose, and 50 μg/mL antibiotics. Quercetin (50 μM) was added at 0, 10, and 18 h (indicated by *arrows*). The culture was incubated at 18 °C. Samples were harvested and analyzed by HPLC

different temperatures for 24 h, equal amounts of cells were used to measure the synthesis of 3-*O*-gentiobioside at 30 °C. The cells (6.2 mg/L) in which protein expression was induced at 18 °C produced more quercetin 3-*O*-gentiobioside than those induced at 30 °C (4.0 mg/L). Although CaUGT was synthesized at higher levels at 30 °C than at 18 °C, the soluble form was synthesized more efficiently at 18 °C. We then tested the optimal cell density for the production of quercetin 3-*O*-gentiobioside. After induction at 18 °C, B-QGB2 cell density was adjusted to OD_{600} of 0.5, 1, 2, 3, and 4, and the *E. coli* was incubated at 30 °C for 7 h. The production of quercetin 3-*O*-gentiobioside increased until an OD_{600} of 3 (from 0.6 mg/L at $OD_{600} = 0.5$, 3.5 mg/L at $OD_{600} = 1$, 5.5 mg/L at $OD_{600} = 2$, and 6.2 mg/L at $OD_{600} = 3$). However, at an OD_{600} of 4, the production decreased to 4.4 mg/L, which was lower than that from a cell density of $OD_{600} = 2$. Next, we tested the reaction temperature. Using the optimized induction time and the cell density for B-QGB2, cells supplemented with 50 μM quercetin were incubated at 18, 25, and 30 °C for 7 h. More quercetin 3-*O*-gentiobioside was produced in cells grown at 18 °C (9.5 mg/L) than at 25 (7.0 mg/L) or 30 °C (6.2 mg/L).

Using the optimized production conditions, we carried out the biotransformation of quercetin into quercetin 3-*O*-gentiobioside. Quercetin (50 μM) was added at 0, 10, and 18 h (to a final concentration of 150 μM) because a higher initial concentration seemed to inhibit the synthesis of quercetin 3-*O*-gentiobioside. Both quercetin 3-*O*-glucoside and quercetin 3-*O*-gentiobioside were produced from the beginning of induction (Fig. 3). The production of quercetin 3-*O*-gentiobioside continued to increase until 33 h, after which it did not significantly increase. The final yield of quercetin 3-*O*-gentiobioside was approximately 46. 2 mg/L (73.8 μM), and 19.8 mg/L (42.5 μM) of quercetin 3-*O*-glucoside was produced. Approximately 77 % of the quercetin was converted into quercetin 3-*O*-glucoside and quercetin 3-*O*-gentiobioside. The conversion of quercetin into quercetin 3-*O*-gentiobioside was approximately 49 %.

Using two UGTs and two nucleotide sugar biosynthesis genes, we could synthesize quercetin 3-*O*-gentiobioside. This compound has been found in some plants (Yan et al. 2014; Ishihara et al. 2016) due to the development of devices. However, the biological activities of this compound have not been explored extensively due to its unavailability. Therefore, the approach used in this study could supply quercetin 3-*O*-gentiobioside to study its biological activities. The approach used in this study could be applied to synthesize diverse flavonoid *O*-diglycosides and a novel biological activity of these compounds can be detected.

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References

An DG, Yang SM, Kim BG, Ahn J-H (2016) Biosynthesis of two quercetin *O*-diglycosides in *Escherichia coli*. J Ind Microbiol Biotechnol 43:841–849

Frydman A, Weisshaus O, Bar-Peled M, Huhman DV, Sumner LW, Marin FR, Lewinsohn E, Fluhr R, Gressel J, Eyal Y (2004) Citrus fruit bitter flavors: isolation and functional characterization of the gene *Cm1,2RhaT* encoding a 1,2 rhamnosyltransferase, a key enzyme in the biosynthesis of the bitter flavonoids of citrus. Plant J 40:88–100

Guardia T, Rotelli AE, Juarez AO, Pelzer LE (2001) Anti-inflammatory properties of plant flavonoids. Effects of rutin, quercetin and hesperidin on adjuvant arthritis in rat. Farmaco 56:683–687

Ishihara H, Tohge T, Viehöver P, Fernie AR, Weisshaar B, Stracke R (2016) Natural variation in flavonol accumulation in Arabidopsis is determined by the flavonol glucosyltransferase BGLU6. J Exp Bot 67:1505–1517

Iwashina T (2003) Flavonoid function and activity to plants and other organisms. Biol Sci Space 17:24–44

Jeandet P, Vasserot Y, Chastang T, Courot E (2013) Engineering microbial cells for the biosynthesis of natural compounds of pharmaceutical significance. BioMed Res Int 2013:13

Jones P, Messner B, Nakajima J-I, Schäffner AR, Saito K (2003) UGT73C6 and UGT78D1, glycosyltransferases involved in flavonol glycoside biosynthesis in *Arabidopsis thaliana*. J Biol Chem 278:43910–43918

Kim B-G, Sung SH, Ahn J-H (2012) Biological synthesis of quercetin 3-*O*-*N*-acetylglucosamine conjugate using engineered *Escherichia coli* expressing UGT78D2. Appl Microbiol Biotechnol 93:2447–2453

Ko JH, Kim BG, Ahn J-H (2006) Glycosylation of flavonoids with a glycosyltransferase from *Bacillus cereus*. FEMS Microbiol Lett 258:263–268

La Casa C, Villegas I, de la Lastra CA, Motilva V, Martín Calero MJ (2000) Evidence for protective and antioxidant properties of rutin, a natural flavone, against ethanol induced gastric lesions. J Ethnopharmacol 71:45–53

Mao Z, Shin HD, Chen RR (2006) Engineering the *E. coli* UDP-glucose synthesis pathway for oligosaccharide synthesis. Biotechnol Prog 22:369–374

Masada S, Terasaka K, Oguchi Y, Okazaki S, Mizushima T, Mizukami H (2009) Functional and structural characterization of a flavonoid glucoside 1,6-glucosyltransferase from *Catharanthus roseus*. Plant Cell Physiol 50:1401–1415

Peterson JJ, Beecher GR, Bhagwat SA, Dwyer JT, Gebhardt SE, Haytowitz DB, Holden JM (2006a) Flavanones in grapefruit, lemons and limes: a compilation and review of the data from the analytical literature. J Food Compos Anal 19:S74–S80

Peterson JJ, Dwyer JT, Beecher GR, Bhagwat SA, Gebhardt SE, Haytowitz DB, Holden JM (2006b) Flavanones in oranges, tangerines (mandarins), tangors, and tangelos: a compilation and review of the data from the analytical literature. J Food Compos Anal 19:S66–S73

Rousseff RL, Martin SF, Youtsey CO (1987) Quantitative survey of narirutin, naringin, hesperidin and neohesperidin in citrus. J Agric Food Chem 35:1027–1030

Sheu JR, Hsiao G, Chou PH, Shen MY, Chou DS (2004) Mechanisms involved in the antiplatelet activity of rutin, a glycoside of the flavonol quercetin, in human platelets. J Agric Food Chem 52:4414–4418

Vogt T, Jones P (2000) Glycosyltransferases in plant natural product synthesis: characterization of a supergene family. Trends Plant Sci 5:380–386

Yan F, Di S, Rodas FR, Torrico TR, Murai Y, Iwashina T, Anai T, Takahashi R (2014) Allelic variation of soybean flower color gene *W4* encoding dihydroflavonol 4-reductase 2. BMC Plant Biol 14:58

Yoon J-A, Kim B-G, Lee WJ, Lim Y, Chong Y, Ahn J-H (2012) Production of a novel quercetin glycoside through metabolic engineering of *Escherichia coli*. Appl Environ Microbiol 78:4256–4262

Zhou K, Qiao K, Edgar S, Stephanopoulos G (2015) Distributing a metabolic pathway among a microbial consortium enhances production of natural products. Nat Biotechnol 33:377–383