Directed evolution and expression tuning of geraniol synthase for efficient geraniol production in *Escherichia coli*

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To achieve an efficient production of geraniol and its derivatives in *Escherichia coli*, we aimed to improve the activity of geraniol synthase (GES) through a single round of mutagenesis and screening for higher substrate consumption. We isolated GES variants that outperform their parent in geraniol production. The analysis of GES variants indicated that the expression level of GES was the bottleneck for geraniol synthesis. Over-expression of the mutant GES<sub>HSV</sub> with a 5′-untranslated sequence designed for high translational efficiency, along with the additional expression of mevalonate pathway enzymes, isopentenyl pyrophosphate isomerase, and geranyl pyrophosphate synthase, yielded 300 mg/L/12 h geraniol and its derivatives (>1000 mg/L/42 h in total) in a shaking flask.

Key Words: high-throughput screening; metabolic engineering; mevalonate pathway; monoterpene; RBS score

Introduction

Geraniol (trans-3,7-dimethyl-2,6-octadien-1-ol) is an acyclic monoterpene alcohol found in plant essential oils. It is commercially important in the flavor and fragrance industries due to its pleasant rose-like odor (Chen and Viljoen, 2010; Rastogi et al., 2001). Geraniol also has value in anticancer drugs (Cho et al., 2016; Lesgards et al., 2014), pesticides, and anthelmintic or antimicrobial reagents (reviewed in Chen and Viljoen, 2010), and it is the precursor of a number of secondary plant metabolites, such as antimitotic alkaloids (Burlat et al., 2004; Geu-Flores et al., 2012). However, geraniol is naturally produced in limited quantities, and considerable effort has been invested in establishing the microbial production of geraniol (Liu et al., 2016; Zhou et al., 2014, 2015).

Geraniol can be synthesized from geranyl pyrophosphate (GPP) by geraniol synthase (GES) (Iijima et al., 2004) (Fig. 1). GPP is derived from two common building blocks, isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), through their head-to-tail condensation. In *Escherichia coli*, this is catalyzed as the first step reaction (EC 2.5.1.1) of the endogenous enzyme IspA. However, this enzyme converts GPP into farnesyl pyrophosphate (FPP) (EC 2.5.1.10), the precursor for ubiquinones, menaquinones, and bactoprenols. Kinetic analysis of IspA (Ku et al., 2005) revealed that it has a lower $K_m$ for GPP than for DMAPP, indicating that GPP could be kinetically ‘channeled’. Due to limited access to the GPP, mere expression of monoterpene synthase resulted only in the production of trace amounts (~μg/L) of monoterpenes (Reiling et al., 2004).

To increase intracellular GPP, researchers have (1) overexpressed enzymes specialized for GPP synthesis (Alonso-Gutierrez et al., 2013; Carter et al., 2003; Reiling et al., 2004), (2) reduced the competing activity of FPP synthases by mutations (Fischer et al., 2011; Ignea et al., 2014; Oswald et al., 2007; Willrodt et al., 2014), and (3) elevated the influx either by over-expressing rate-limiting enzymes of the methylerythritol 4-phosphate (MEP) pathway or by introducing exogenous MEV pathways. Collectively, these efforts, along with the development/optimization of fed-batch/high-density fermentation processes, have enabled the production of 1–2 g of geraniol per liter of medium (Liu et al., 2016; Wu et al., 2015). Nevertheless, attainable production efficiency remains far less than that for hemiterpenes (Whited et al., 2010) and sesquiterpenes...
We ligated the resulting PCR fragment into the vector used lose membranes (BioTrace NT Nitrocellulose Transfer) as an inducer of GES expression) with nitrocellulose and was plated onto LB-Lennox agar plates to form colonies. These colonies were picked and inoculated into 500 μL of LB-Lennox medium in a 96-deep-well plate and cultured at 37°C, 1000 rpm, for 12 h. An aliquot (20 μL) of these pre-cultures was transferred to 2 mL of Terrific broth (TB) containing 0.02% (w/v) arabinose in a 48-deep-well plate and cultured at 30°C, 1000 rpm for 48 h. The cells were harvested, washed with saline, and centrifuged to obtain cell pellets, and the supernatants were discarded. One mL of acetone was added to each of the pellets, and they were vortexed for 1 min to extract the carotenoids. This was followed by centrifugation. Next, the absorbance spectra (450–600 nm at 5-nm intervals) of the supernatants were analyzed by using a SpectraMax Plus Absorbance Microplate Reader (Molecular Devices, Sunnyvale, CA). The pigmentation level of each culture was determined based on the lambda max of the resulting extract by using the molar adsorption coefficients of diaponeurosperone (470 nm, 147,000 M⁻¹ cm⁻¹).

Materials and Methods

Strains and plasmids. XL1-Blue (recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F’ proAB lacF’ZM15 Tn10 (Tet’)]) (Stratagene) was used for DNA cloning, library construction, and terpene production. E. coli BW25113Δmaa (Δ araD-araB)567 Δ lacZ4787::(::rrnB-3 Δ tnaA739::kan rph-l Δ(araD-araB)567, hsdR514) from the KEIO collection (Baba et al., 2006) was used for screening and carotenoid production.

Antibiotics were added at the following concentrations: 50 μg/mL, carbenicillin (Sigma-Aldrich, St. Louis, MO), 30 μg/mL, chloramphenicol (Nacalai Tesque, Kyoto), and/or 30 μg/mL kanamycin (Sigma-Aldrich).

To construct pAC-MN-idi, the region encoding the lac operon was PCR-amplified from pUC-idi, which was achieved by inserting an E. coli idi gene into the Xbal/Xhol site of a pUC18m vector, and then ligating it into the Sall site of pAC-MN (Furubayashi et al., 2014).

pUCara-GES M53 was constructed by PCR-amplifying the reading frame of GES M53 from pUC-GES M53 (Furubayashi et al., 2014) and subcloning it into the EcoRI site of XL1-Blue harboring pBBRSOE6 (Reiling et al., 2004) or co-transformed with pMEV-idi-GPPS (Tashiro et al., 2016) into XL1-Blue. The colonies were inoculated into 500 μL of LB-Lennox medium in a 96-deep-well plate and cultured at 37°C, 1000 rpm for 16 h. An aliquot of these pre-cultures was transferred (1:100 dilution) into TB medium containing 2% (v/v) glycerol (as a carbon source) in a flask and cultured at 30°C (for pBBRSOE6 strains) or 37°C (for pMEV-idi-GPPS strains), 200 rpm until the OD₆₀₀ reached 0.4–0.6. For pBBRSOE6 strains, 0.02% (w/v) arabinose was added to induce the genes, and the mixture was cultured for an additional 8–12 h. Note that there was no detectable loss of geraniol from the culture in this experiment (Supplementary Fig. S1). For pMEV-idi-GPPS strains, after 0.02% (w/v) arabinose and 10 μM IPTG were added to induce the genes, 2 mL (20% (v/v) of the original culture) of decane was overlaid onto the cultures, and they were allowed to shake for an additional 12–42 h at 30°C.

To directly extract the geraniol from the culture, aliquots (1.5 mL) of the culture were collected in 2-mL tubes, and 300 μL of ethyl acetate was spiked with an internal standard of (S)-(−)-limonene (Sigma-Aldrich) before being added to the samples, which were then vortexed for 20 s. After a short centrifugation, the organic phase was collected and analyzed by using a GC-FID (Shimadzu Corporation, Kyoto) equipped with an Rtx5-ms capillary column (30 m x 0.25 μm ID and 0.25-μm film thickness, Restek). Splitless injections (1 μL) were performed with an injector and an FID detector temperature of 250°C, and the samples were separated on a GC with an oven temperature programmed to start at 60°C for 3 min; this was increased by 6°C min⁻¹ up to 150°C, followed by an increase of 15°C min⁻¹ until the temperature reached 230°C. To quantify the geraniol in the decane phase, 10 μL of the decane fraction was collected and di-
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luted in 990 mL of ethyl acetate spiked with limonene as an internal standard. GC analysis was performed as previously reported (Tashiro et al., 2016). For quantification, the calibration curve was drawn with a geraniol standard purchased from Sigma-Aldrich.

**Western blot analysis.** pUCara-GES variants were transformed into XL1-Blue. The colonies were inoculated into 500 μL of LB-Lennox medium in a 96-deep-well plate and cultured at 37°C, 1000 rpm for 16 h. An aliquot of these pre-cultures was transferred (1:100 dilution) into TB medium in a flask and cultured at 30°C, 200 rpm until the OD<sub>600</sub> reached 0.4–0.6. Then, 0.2% (w/v) arabinose was added to induce the genes, and the mixture was cultured for an additional 4 h. The culture was transferred into two 2-mL tubes and centrifuged to obtain cell pellets. One of the cell pellets was resuspended with 10 μL of sample buffer (glycerol, SDS, stacking gel buffer, BPB, 2-mercaptoethanol) to prepare the total fraction. Another cell pellet was resuspended in 50 μL of lysis buffer (50 mM Tris-HCl, 150 mM NaCl, and 1 mg/ml lysozyme (Sigma-Aldrich)), and lysed by freeze-thaw. The lysate was centrifuged at 13,000 rcf for 30 min, and 10 μL of its supernatant was taken and diluted in 2× volume of sample buffer as the soluble fraction. The total and soluble fractions were subjected to dodecyl sulfate polyacrylamide gel electrophoresis. The proteins were transferred from the polyacrylamide gel to nitrocellulose membranes (BioTrace NT Nitrocellulose Transfer Membrane, Pall Corporation) using the semi-dry blotting transfer system. The membrane was blocked with 5% skim milk in PBS containing Tween 20 for 1 h and then washed and incubated with HRP-conjugated 6× His antibody (6× His mAb-HRP Conjugate, Clontech, Mountain View, CA) in PBS for 1 h at room temperature. Finally, the membranes were washed with PBS and stained with diaminobenzidine.

**Results**

**Expression system for GES mutants**

Previously, we constructed a series of N-truncated mutants of GES to remove a transit-peptide sequence and to improve enzyme solubility (Furubayashi et al., 2014). The mutant GES<sub>53</sub>, which lacks the first 53 amino acids, turned out to be the best geraniol producer in *Escherichia coli*. In this work, we aimed to further evolve GES<sub>53</sub> to obtain higher-performing variants of geraniol synthase. Most likely, due to the cytotoxicity of GES<sub>53</sub>, *E. coli* constitutively expressing GES<sub>53</sub> from a plasmid yielded small and uneven colonies. This is highly unfavorable for the subsequent screening step, where one must discriminate between the various expression systems used.
nate subtle differences in the pigmentation among the colonies. By placing \( \text{GES}_{M53} \) under a stringent araBAD promoter (Khlebnikov et al., 2000), we managed to establish robust growth conditions both for colony screening and for the efficient production of geraniol (described in the following sections).

**Screening for higher geraniol producer**

Recently, we reported a high-throughput screening method for the cellular activity of terpene synthases (Furubayashi et al., 2014). The method is based on the indirect visualization of precursor consumption for each synthase variant. Scores are based on the color change of the cell due to the decrease in the yields of co-existing carotenoid pathways (Fig. 1). Although this method was demonstrated to be widely applicable for various terpene synthases, including GES, we had to change the system to further evolve it. With the original pAC-MN (Fig. 2, upper panels), carotenoid-producing strains of \( E.\ coli \) undergo a nearly complete loss of cell pigmentation when they express \( \text{GES}_{M53} \). This indicates that \( \text{GES}_{M53} \) is already too active, such that its precursor is virtually inaccessible to the carotenoid pathway.

To identify variants with even higher activities, we must elevate the relative expression levels of carotenoids or increase the precursor supply so that some of the resources become accessible to the carotenoid biosynthetic pathway. We found that overexpression of isopentenyl pyrophosphate isomerase (Idi, 5.3.3.2), which has long been known to increase the production levels of terpenes and carotenoids (Kajiwara et al., 1997). Replacing pAC-MN with pAC-MN-idi, pigmentation in cells expressing carotenoid pathway enzymes were restored even in the presence of \( \text{GES}_{M53} \) (Fig. 2, lower panels).

**Directed evolution of \( \text{GES}_{M53} \)**

The entire reading frame of \( \text{GES}_{M53} \), together with the 5’-untranslated regions, including the ribosome binding site, was subjected to error-prone PCR (Cirino et al., 2003), and the amplified DNA fragment was sub-cloned into the

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**Fig. 3.** Experimental procedure for the directed evolution of GES for higher cellular activity.

**Fig. 4.** Carotenoid production of \( E.\ coli \) cells harboring GES variants and pAC-MN-idi. The data are presented as the average of three independent experiments, with error bars representing the standard deviation from the mean.
plasmid under the control of an arabinose-inducible promoter (pBAD/araC).

An E. coli strain harboring plasmid pAC-MN-idi was subjected to a second transformation with the plasmid library of pUCara-GESM53. Note that the E. coli strain we chose (BW25113ΔtntA) was deficient in araB, the arabinoose (inducer) consumer enzyme (AraB), thus assuring the prolonged maintenance of the “induced” state (Morgan-Kiss et al., 2002).

Approximately 3,000 individual transformant cells were grown on nitrocellulose sheets on LB-agar plates including arabinose (inducer) to form colonies (Fig. 3). After another 8 h at room temperature, the colonies were individually scored for their ‘yellowness’. To score them, we took pictures of the colonies using a flatbed scanner, separated the RGB channels of the images, and used the intensity value of the blue channel as the ‘score’ of each clones. We found that as many as 150 clones (2–5% of the pool) exhibited colonies with weaker pigmentation that those expressing their parent, GESM53.

**Second screening of GES variants**

We isolated plasmids encoding GES mutants from 30 representative colonies with low pigmentation, and fresh cells expressing pAC-MN-idi were transformed with each plasmid. Thirteen mutants formed colonies with a pale color, whereas the others did not appear significantly different from the cells expressing GESM53.

To confirm the improvement in substrate consumption, the cells expressing the thirteen GES variants were tested for their carotenoid production. The expression of GESM53 (parental clone) resulted in the accumulation of 160 µg/g of carotenoid pigments per dry cell weight (gDCW hereafter), which is approximately 80 µg/gDCW lower than the cells expressing the inactivated mutant of GESM53 (GESM53(D323)) (Fig. 4). When transformed with GES mutants, the cells accumulated even lower amounts of carotenoid (50–120 µg/g-DCW). This indicates that they have higher substrate consumption than their parent GESM53.

**GES variants exhibited higher geraniol production**

Five selected GES variants were individually re-transformed into the cell not harboring a carotenoid pathway, and the geraniol accumulated in the media was quantitated with GC-FID.

To improve the reliability of the assay, we moderately elevated the precursor-supplying capacity of the host cell by introducing the plasmid pBBRSOE6, which was developed by Reiling et al. (2004). This plasmid elevates the cellular levels of GPP by constitutively expressing three enzymes: the rate-limiting enzyme Dxs (1-deoxy-d-xylulose 5-phosphate synthase) of the endogenous precursor pathway (MEP pathway), Idi from Haemococcus pluvialis, and the GPP-synthesizing mutant of FPP synthase (IspAθ395). All of the tested GES mutants exhibited significantly higher geraniol production than GESM53, confirming that the isolates were higher-performing (Fig. 5). More importantly, we observed a nearly perfect linear and negative correlation between the production levels of geraniol and those of carotenoids co-existing in the cell (Fig. 5). Thus, carotenoid screening is a rapid yet highly reliable predictor of the cellular performance of geraniol synthase. We expect that the same should be true for a variety of other monoterpene synthases as well.

**GES variants exhibited an improved translation initiation rate**

Given the unusually high frequency of positive clones (2–5% of the entire population), as well as the general rarity of mutations that confer biochemical improvements in enzymes, we assumed that most of the GES mutants with improved cellular activity would be ‘expression mutants’ carrying mutations that improve their expression levels.

Sequence analysis of the GES variants revealed that this was, in fact, the case: all of them possessed at least one nucleotide substitution in the 5′-untranslated regions or at positions very close to the translation initiation site (ribosome binding site, RBS hereafter) (Fig. 6). It has been well recognized that the sequences neighboring the RBS/ start codons, including the first ~38 nucleotides of the reading frame, significantly alter the expression level of the encoded protein (Kudla et al., 2009). For each GES variant, we calculated a score for translation initiation, which we present as “RBS scores” using the state-of-the-art designing program called RBS calculator (Salis et al., 2009) (Fig. 6). We found that the majority of the GES variants (12 out of 14) had 1.2- to 8.2-fold higher RBS scores (966–6946) than their parent, GESM53 (844).

Western blotting further confirmed this hypothesis (Fig. 7): all of the four tested GES variants, including the two variants (GESmut8 and GESmut9) with lower calculated RBS scores, turned out to have a higher expression level.

**Forward engineering of RBS for higher expression**

Given that the cellular performance of GES can be improved by mutations that alter the translation initiation efficiency, we decided to maximize the expression level of GESM53 by further elevating the translation initiation efficiency. With the aid of the “forward engineering mode”
in the RBS calculator, we designed and synthesized three additional constructs to express GESM53 with scores of 40,258 (GES mut14), 457,402 (GES mut15), and 1,957,076 (GES mut16) (for the sequence, see Fig. 6). We observed that the expression level of GES increased with increasing RBS score, but it became saturated at an RBS score of 40,258 (Fig. 8A). Beyond this score, the cell may be limited by translational resources (ribosomes and acyl-tRNAs). Alternatively, the effective concentration of GESM53 in the cell may have reached the maximum solubility.

**Geraniol production in the designed GES M53 expression system**

We tested the performance of our GES expression system. To increase the precursor supply, we first tried co-introducing the plasmid pBBRSOE6 (Reiling et al., 2004), which expresses Dxs, Idi, and IspAS80F (see above). As the RBS scores increased, the amount of geraniol excreted by the cell expressing GES M53 increased. Interestingly, the geraniol production plateaued at 10 mg/L, with a score of 4,667 (Fig. 8B). This occurred despite the fact that the expression of GES is significantly higher in cells harboring the GES M53-expressing plasmid with an RBS score of 40,258 (Fig. 8A). Beyond this score, the cell may be limited by translational resources (ribosomes and acyl-tRNAs). Alternatively, the effective concentration of GES M53 in the cell may have reached the maximum solubility.

**Accumulation of geraniol-derived compounds**

Geraniol can be dehydrogenated (Zhou et al., 2014), isomerized (Akhila, 1985), reduced (Fischer et al., 2013), or acetylated (Zhou et al., 2014), in *E. coli* to yield various geraniol-derived compounds. Our culture also contained a variety of geraniol-related compounds (Figs. 8B and C). After 12 h of shaking incubation, they (geranyl acetate, geranial) were observed only as minor components (ca. 16 and 14%, respectively), and geraniol was the main product (ca. 70%).

After extended incubation times, however, the accumulation of these byproducts greatly increased (Fig. 9); the level of geraniol remained at 300 mg/L, but a complex mixture of geraniol-derived compounds increased over time, approaching 900 mg/L in total. At 42 h, we observed more than 1 g/L of geraniol and its derivatives.
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Fig. 8. Forward engineered GES-expression plasmids and their performance in geraniol production.

A. Western blotting analysis of GES variants. B. Geraniol production by GES<sub>M53</sub> with designed RBSs, with the engineered MEP pathway. C. Geraniol production by GES<sub>M53</sub> with the additional expression of the heterologous MEV pathway. For B and C, values given by the RBS calculator (Salis et al., 2009) were the approximated RBS translation efficiency. All data are the averages of three independent experiments, with error bars representing the standard deviation from the mean. Filled circles indicate the total amounts of geraniol (open circle), citronellol and nerol (open triangle), geranylacetate (open square), and geranial (open diamond).

Discussion

The mutant GES<sub>M53</sub> was previously isolated from N-truncated libraries of GES and shows increased activity in the heterologous host (*E. coli*). By optimizing its translation initiation efficiency, increasing the precursor pool, and adopting a two-phase culture system, we achieved a production of >1,000 mg/L/42 h of geraniol and its derivatives in a shaking flask of *E. coli* culture. The current record for monoterpen synthesis is twice this value (2,000 mg of geraniol /L/24 h (Liu et al., 2016)), but it should be noted that this was achieved in a high-density fed-batch reactor (OD<sub>600</sub> ~ 32, more than five times in cell density compared with our flask culture with maximum OD<sub>600</sub> ~ 5).

To further improve the production of geraniol, it will be
important to block its conversion into byproducts. The acetylation of geraniol is known to be catalyzed by the promiscuous activity of chloramphenicol acetyltransferase (Zhou et al., 2014), which is included as a selection marker on the booster plasmid that we used. We should be able to significantly reduce this activity by simply exchanging the selection marker of pMEV-GPPS-idi for the kanamycin resistance gene. It was recently reported that the enzyme YigB is responsible for oxidizing geraniol to produce geranial (Zhou et al., 2014), and therefore knocking out this gene should be effective in trimming this pathway. The catalytic source of citronellol biosynthesis remains, as yet, unclear. In yeast, the enzyme (old yellow enzyme) responsible for the reduction of geraniol has been identified (Steyer et al., 2013). We deleted its homolog, nemA, but did not observe a decrease in citronellol production (Tashiro, M., unpublished data). We believe that there are other enzymes that act on geraniol.

In our engineering efforts, we found that the expression level of GESM53 is the bottleneck of our geraniol production system (Fig. 8C). This holds true even when the cellular environment is saturated with GESM53, and despite the fact that GES is one of the most active enzymes ($k_{cat} = 0.8$ s$^{-1}$) (Iijima et al., 2004) for a terpene synthase [EC 4.2.3.11]. It would be interesting to test whether there is still room to improve the enzymatic properties of GES.

Unlike plants, E. coli lacks a specific GPP synthase, and GPP is synthesized as the intermediate of the FPP synthase, ispA. It has been known that GPP is immediately converted to FPP, releasing only a trace amount of free GPP that is accessible to monoterpene synthases. Therefore, reinforcement of GPP to monoterpene activity is key to the efficient production of monoterpenes. To further improve the production level of monoterpenes, our finding that monoterpene synthase is the bottleneck suggests that terpenoid synthases must be re-engineered to have higher catalytic activity (Tashiro et al., 2016) or improved solubility/stability in the heterologous environment.

Directed evolution is a powerful tool and is virtually the only available technology to improve the catalytic efficiency of enzymes. To enable this for terpene synthases, we have developed a colony-based screening method to identify terpene synthase mutants with a higher capability to consume their substrates (Furubayashi et al., 2014). Although this method quickly identified the highly active variants of GES (GESM53) from a truncation library, the activities scored by this screening system were saturated (near-complete interception of precursor available for pigment biosynthesis), thus hampering the further search for improvements in activities. In the present study, we have shown that over-expression of isopentenyl pyrophosphate isomerase (Idi) is effective in moderately increasing the GPP pool available for carotenoid biosynthesis, thereby enabling the search for improved geraniol synthase activity in the cell. Exceptional linearity between the pigment score and actual cellular performance (Fig. 5) indicates that this screening principle is reliable. By gradually increasing the precursor pool within the cell, it should be possible, at least theoretically, to keep evolving the improved monoterpene synthases in a step-by-step manner.

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Supplementary Materials

Supplementary figure is available in our J-STAGE site (http://www.jstage.jst.go.jp/browse/gjam).

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