Enzyme and Microbial Technology 68 (2015) 50–55

Contents lists available at ScienceDirect

Enzyme and Microbial Technology

journal homepage: www.elsevier.com/locate/emt

Geranyl diphosphate synthase: An important regulation point in balancing a recombinant monoterpene pathway in *Escherichia coli*

Jia Zhou[a,1], Chonglong Wang[a,1], Liyang Yang[a], Eui-Sung Choi[b,**], Seon-Won Kim[a,*]

[a] Division of Applied Life Science (BK21 Plus), PMBBRC, Gyeongsang National University, Jinju 660-701, Republic of Korea
[b] Industrial Biotechnology Research Center, KRIBB, Daejeon 305-806, Republic of Korea

ARTICLE INFO

Article history:
Received 28 May 2014
Received in revised form 16 October 2014
Accepted 17 October 2014
Available online 25 October 2014

Keywords:
Escherichia coli
Geraniol
GPP synthesis
Mevalonate pathway
Plasmid maintenance
RBS strength

ABSTRACT

The expression level of geranyl diphosphate synthase (GPPS) was suspected to play a key role for geraniol production in recombinant *Escherichia coli* harboring an entire mevalonate pathway operon and a geraniol synthesis operon. The expression of GPPS was optimized by using ribosomal binding sites (RBSs) designed to have different translation initiation rates (TIRs). The RBS strength in TIR window of 500 arbitrary unit (au)–1400 au for GPPS appears to be suitable for balancing the geraniol biosynthesis pathway in this study. With the TIR of 500 au, the highest production titer of geraniol was obtained at a level of 1119 mg/L, which represented a 6-fold increase in comparison with the previous titer of 183 mg/L. The TIRs of *GPPS* locating out of range of the optimal window (500–1400 au) caused significant decreases of cell growth and geraniol production. It was suspected to result from metabolic imbalance and plasmid instability in geraniol production by inappropriate expression level of GPP synthase. Our results collectively indicated GPPS as an important regulation point in balancing a recombinant geraniol synthesis pathway. The GPPS-based regulation approach could be applicable for optimizing microbial production of other monoterpenes.

1. Introduction

Over the past two decades, microbial engineering has been emerged as a promising tool for efficient and sustainable production of valuable chemicals from renewable biomass. Geraniol (*trans*-3,7-dimethyl-2,6-octadien-1-ol; $C_{10}H_{18}O$) is a monoterpene alcohol with economic importance in food chemistry, pharmacy and cosmetic industry [1], and even the fast developing biofuel industry [2–4]. Geraniol can be synthesized by geraniol synthase from geranyl diphosphate (GPP), the universal precursor of monoterpenes. GPP synthase (GPPS) catalyzes the formation of GPP through the head-to-tail condensation of dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP) generated from either the mevalonate (MVA) pathway or the methylerythritol phosphate (MEP) pathway [5,6] (Fig. 1A). The genetically tractable *E. coli* host was engineered to heterologously express geraniol synthase, GPPS and the entire MVA pathway for geraniol production where the highest titer of 183 mg/L has been obtained in our previous study [7].

Metabolic engineering for production of foreign compounds often suffers from low yields due to various obstacles, such as the promiscuous metabolic activity of host hampering foreign metabolic product formation [7], and the metabolic imbalance imposing burden on host [8]. In the previous study we have shown that geraniol production was significantly increased by reducing the endogenous dehydrogenization and isomerization of geraniol [7]. Nevertheless, the geraniol production is also likely challenged by metabolic imbalance of isoprenyl diphosphate synthesis. Isoprenyl diphosphates are suggested to be toxic when accumulated in cells [9], but on the other hand, they are also essential cellular metabolites for synthesis of ubiquinones [10], cell walls [11], and tRNA [12]. Therefore, a balanced metabolism (neither over-accumulation nor depletion) of IPP/DMAPP and GPP could be critical for the improved production of monoterpenes in recombinant strains. Since GPPS is responsible for conversion of IPP/DMAPP to GPP, we have performed optimization of geraniol synthesis pathway by carefully modulating the expression level of GPPS.

* Corresponding author. Tel.: +82 55 772 1362; fax: +82 55 759 9363.
** Corresponding author. Fax: +82 42 860 4489.
E-mail addresses: choi4162@kribb.re.kr (E.-S. Choi), swkim@gnu.ac.kr (S.-W. Kim).
[1] Both authors contributed equally to this paper.

http://dx.doi.org/10.1016/j.enzmictec.2014.10.005
0141-0229/

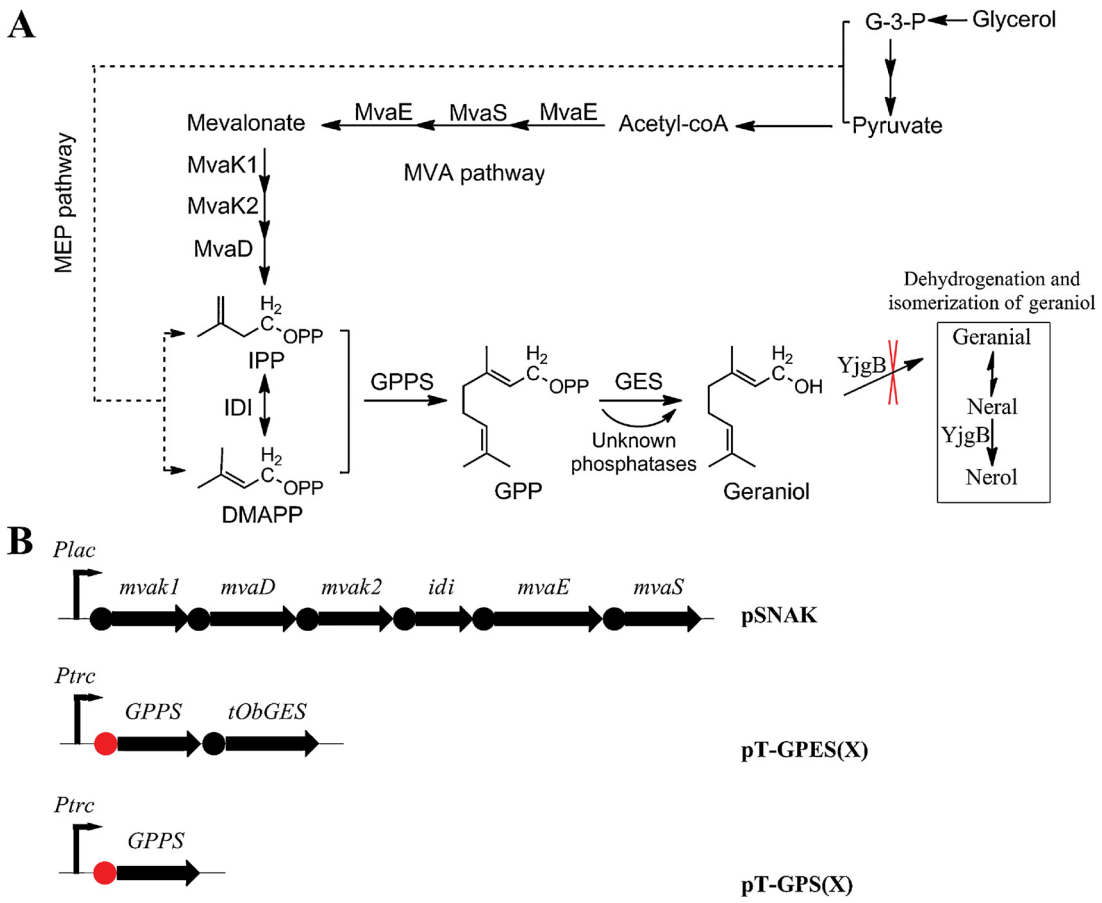

Fig. 1. Schemes of the geraniol biosynthesis pathway in engineered *E. coli* and the corresponding operons. (A) Geraniol biosynthesis pathway in engineered *E. coli*. Building blocks IPP and DMAPP are synthesized from both the native MEP pathway (dashed arrow) and the heterologous MVA pathway using glycerol as the main carbon source. GPP is synthesized from IPP and DMAPP by GPPS. GPP is finally converted to geraniol by geraniol synthase (GES) and unknown endogenous phosphatases. The dehydrogenation of geraniol to geranial is blocked by deletion of gene *yjgB*. The blocked pathway was indicated with a red cross. Abbreviations of the pathway intermediates and enzymes are as follows: G-3-P, glyceraldehyde 3-phosphate; DMAPP, dimethylallyl diphosphate; IPP, isopentenyl diphosphate; GPP, geranyl diphosphate; MvaE, bifunctional acetoacetyl-CoA thiolase and HMG-CoA reductase; MvaS, HMG-CoA synthase; MvaK1, mevalonate kinase; MvaK2, phosphomevalonate kinase; MvaD, mevalonate diphosphate decarboxylase; IDI, IPP isomerase; GPPS, geranyl diphosphate synthase; and GES, geraniol synthase. (B) MVA pathway operon and geraniol synthesis operons. Solid arrows, solid circles, and bent arrows represent genes, RBS, and promoters, respectively. The red solid circle represents RBSs with different translation initiation rates. All genes are corresponding to the enzymes shown in (A), and tObGES represents a truncated form of geraniol synthase from *Ocimum basilicum*. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2. Materials and methods

2.1. Strains and culture conditions

Strains used in this study are listed in Table 1. All experiments, including gene manipulation, seed preparation and production culture, were conducted in 2YT medium (16 g tryptone, 10 g yeast extract, and 5 g sodium chloride per liter) containing 2% glycerol as a main carbon source. *E. coli* DH5α used for plasmid construction was grown at 37 °C overnight, while strains used for seed and production cultures were cultivated at 30 °C. Antibiotics were added at a concentration of 100 mg/L for ampicillin and 50 mg/L for kanamycin, respectively. All geraniol production cultures were inoculated in an initial optical density of 0.4–0.6 at 600 nm (OD_{600}) with a seed from log phase of growth. Cultures were initially induced with 1 mM of IPTG. To harvest the geraniol produced during culture, a two-phase culture was carried out by overlaying 1 mL of decane over 5 mL of culture broth [7].

2.2. Plasmids and constructions

Polymerase chain reaction (PCR) primers and plasmids used in this study are listed in Table 1. Ribosomal binding site (RBS) sequences with different translation initiation rates (TIRs) were designed using Salis Lab's RBS Calculator [13]. The RBS sequences with TIRs of 100 arbitrary unit (au), 200 au, 500 au, 1400 au and 5300 au were added to 5′-end of *GPPS* by using PCR with forward primers of GPPS(0.1K)-F, GPPS(0.2K)-F, GPPS(0.5K)-F, GPPS(1.4K)-F and GPPS(5.3K)-F, and reverse primer of GPPS-BmI-R along with the template of plasmid pT-GPS (Table 1). The various amplified *GPPS* products were then inserted between *Eco*RI and *Bam*HI restriction sites of pTrc99A vector [14], resulting in the plasmids of pT-GPS(0.1K), pT-GPS(0.2K), pT-GPS(0.5K), pT-GPS(1.4K), and pT-GPS(5.3K). Geraniol synthase gene *tObGES* was digested from pT-GPSGES [7] with *Bam*HI and *Sal*I and inserted in the plasmids of pT-GPS(0.1K), pT-GPS(0.2K), pT-GPS(0.5K), pT-GPS(1.4K) and pT-GPS(5.3K) to construct a set of geraniol synthesis plasmids, pT-GEOL(0.1K), pT-GEOL(0.2K), pT-GEOL(0.5K), pT-GEOL(1.4K) and pT-GEOL(5.3K), respectively.

2.3. Protein expression analysis

For analysis of GPPS expression level dependent on its TIRs, the plasmids of pT-GPS(X; X = 0.1K, 0.2K, 0.5K, 1.4K and 5.3K) and pT-GPS (Table 1) were transformed into strain MGΔ*yjgB*, and overexpressed under the culture condition of geraniol production. Equal amounts of cells (based on OD_{600} cell density) were collected from 12 h of cultures and re-suspended in 300 μL lysis buffer (10 mM Tris–Cl, pH8.0, 1 mM EDTA, and 1% Triton X-100). The suspensions were sonicated on ice to lyse cells and centrifuged at 12,000 rpm for 25 min [15]. The resulted supernatants containing the soluble cytosolic proteins fractions were loaded to 12% SDS-PAGE with equal volumes for the analysis.

2.4. Plasmid maintenance monitoring

Production cultures under antibiotic selective pressures were taken at 12 h of culture after IPTG induction, diluted with 2YT medium to a proper OD_{600}, and spread on LB (Luria–Bertani) agar plates with no antibiotic, with ampicillin (100 mg/L), with kanamycin (50 mg/L), and with both ampicillin and kanamycin, respectively, for overnight growth. The colony number was restricted within a range between 100 and 500. Plasmid maintenance (%) is estimated by the colonies survived on different antibiotics-containing plates.