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Engineering *Escherichia coli* for selective geraniol production with minimized endogenous dehydrogenation

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ABSTRACT

Geraniol, a monoterpene alcohol, has versatile applications in the fragrance industry, pharmacy and agrochemistry. Moreover, geraniol could be an ideal gasoline alternative. In this study, recombinant over-expression of geranyl diphosphate synthase and the bottom portion of a foreign mevalonate pathway in *Escherichia coli* MG1655 produced 13.3 mg/L of geraniol. Introduction of *Ocimum basilicum* geraniol synthase increased geraniol production to 105.2 mg/L. However, geraniol production encountered a loss from its endogenous dehydrogenization and isomerization into other geranoids (nerol, neral and geranial). Three *E. coli* enzymes (YjgB, YahK and YddN) were identified with high sequence identity to plant geraniol dehydrogenases. YjgB was demonstrated to be the major one responsible for geraniol dehydrogenization. Deletion of yjgB increased geraniol production to 129.7 mg/L. Introduction of the whole mevalonate pathway for enhanced building block synthesis from endogenously synthesized mevalonate improved geraniol production up to 182.5 mg/L in the yjgB mutant after 48 h of culture, which was a double of that obtained in the wild type control (96.5 mg/L). Our strategy for improving geraniol production in engineered *E. coli* should be generalizable for addressing similar problems during metabolic engineering.

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1. Introduction

Geraniol (trans-3,7-dimethyl-2,6-octadien-1-ol; $C_{10}H_{18}O$) is an acyclic monoterpene alcohol found in plant essential oils. It is commercially important in flavor and fragrance industries due to its pleasant rose-like odor (Rastogi et al., 2001). Geraniol also exhibits good prospectives in pharmacy and agrochemistry as anticancer drugs (Carnesecchi et al., 2004; Polo et al., 2011), antimicrobial reagents (Togashi et al., 2008; Unlu et al., 2010) and biopesticides (Barnard and Xue, 2004; Papachristos et al., 2004). Besides, geraniol is considered a gasoline alternative superior to ethanol due to its low hygroscopicity, high energy content, and relatively low volatility (Peralta-Yahya and Keasling, 2010).

Geraniol is biosynthesized by geraniol synthase from geranyl diphosphate (GPP) which is the universal precursor of monoterpenes. GPP is synthesized by GPP synthase (GPPS) via head to tail condensation of isopentenyl diphosphate (IPP) with dimethylallyl diphosphate (DMAPP) which can be produced from either the mevalonate (MVA) pathway or the methylerythritol phosphate (MEP) pathway (Goldstein and Brown, 1990; Rohmer, 1999). Although microorganisms do not normally accumulate GPP, mutations in farnesyl diphosphate synthase (FPPS) allow GPP release for monoterpene biosynthesis in recombinant microorganisms harboring monoterpene synthases (Fischer et al., 2011; Oswald et al., 2007; Reiling et al., 2004). GPP accumulation in yeast bearing mutated FPPS enabled geraniol formation in the absence of a heterologous geraniol synthase probably through endogenous dephosphorylation (Blanchard and Karst, 1993; Fischer et al., 2011; Oswald et al., 2007). Similarly, other terpenoid alcohols, such as farnesol and geranylgeraniol, could also be produced in engineered microbes over-expressing FPPS or geranylgeranyl diphosphate synthase (GGPPS) (Muramatsu et al., 2008; Ohto et al., 2009; Tokuhiro et al., 2009; Wang et al., 2010). Thus, it is of interest to see whether geraniol can be produced in the absence of geraniol synthase in a GPP-synthesizing *Escherichia coli* strain. A recent study demonstrated that geraniol could be generated at a level of 0.185 mg/L even in the absence of specific GPPS or mutated FPPS in *E. coli* by simply over-expressing a *Ocimum basilicum* geraniol synthase (ObGES), although the GPP release mechanism remained unclear (Fischer et al., 2013). By co-overexpression of a FPPS mutant and the ObGES in *Saccharomyces cerevisiae*, geraniol production was increased to 5 mg/L after 7 days of culture (Fischer et al., 2011). So

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far, the highest geraniol production has been obtained at a level of 36.04 mg/L in *S. cerevisiae* harboring both FPPS mutant and ObGES after 48 h of culture by overexpressing key rate-limiting enzymes of the mevalonate pathway (Liu et al., 2013). However, this titer is too low to apply to industrial processes. The limited production of geraniol thus prompted us to engineer an effective geraniol biosynthesis pathway in *E. coli*.

Previous studies have individually or partially examined three critical points for increasing geraniol production: increases of building blocks IPP and DMAPP synthesis, GPP synthesis, and GPP conversion to geraniol (Blanchard and Karst, 1993; Fischer et al., 2011, 2013; Liu et al., 2013; Oswald et al., 2007). In this study for maximization of geraniol production, we combined all three aspects and collectively engineered *E. coli* to have an exogenous MVA pathway for increasing building block synthesis, a GPPS mutated from *E. coli* FPPS for GPP synthesis, and a truncated geraniol synthase from *O. basilicum* for efficient conversion of GPP to geraniol (Fig. 1). In addition, we observed endogenous dehydrogenation and isomerization of geraniol in *E. coli*. The geraniol dehydrogenation and isomerization pathway was thus investigated and blocked to further increase geraniol production.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Strains used in this study are listed in Table 1. FRT-flanked kanamycin resistance cassettes amplified from plasmid pKD13 with primer sets yjgB-KO-F/yjgB-KO-R, yddN-KO-F/yddN-KO-R, and yahK-KO-F/yahK-KO-R were used for establishing knock-out strains, MGΔ*yjgB*, MGΔ*yddN* and MGΔ*yahK*, respectively, with a one-step recombinant method (Baba et al., 2006; Datsenko and Wanner, 2000). Kanamycin resistance markers were eliminated from the chromosomes of the knock-out strains according to the reported method (Datsenko and Wanner, 2000). The non-kanamycin resistant mutants were confirmed by PCR using primer sets YjgB-P-F/yjgB-KO-R, YddN-P-F/yddN-KO-R, and YahK-P-F/yahK-KO-R, respectively. Antibiotics with appropriate concentrations (100 mg/L ampicillin and 50 mg/L kanamycin) were added as required in experiments. Glycerol stocks were streaked out on solid LB medium (10 g tryptone, 10 g sodium chloride, 5 g yeast extracts, and 17 g agar per 1 L) and grown overnight. Glass tube (2.5 cm × 15 cm) with plastic cap was used for aerobic culture of the seed and main fermentation. To make seed cultures, individual colonies were picked to inoculate 5 ml 2YT medium (16 g tryptone, 5 g sodium chloride, and 10 g yeast extracts per 1 L) containing 2% glycerol as the main carbon source, and cultured at 30 °C and 250 rpm overnight. Seed from log phase of growth was then inoculated into 5 ml of the same fresh medium to make an initial optical density at 600 nm (OD_{600}) of 0.1 for geranoid feeding experiments, and of 0.4–0.6 optimized for geraniol production. To harvest geranoid from the culture broth during fermentation, two-phase culture was carried out by overlaying 1 ml decane over 5 ml culture broth (Wang et al., 2010). The cultures for geraniol production were performed at 30 °C and 250 rpm for 48 h with initial supplementation of 1 mM IPTG. Mevalonate was prepared from mevalonolactone (Sigma-Aldrich; CAS No. 674-26-0) as described in a previous report (Kim et al., 1992), and initially fed at a concentration of 3.2 mM for culturing of *E. coli* strains harboring the bottom portion of MVA pathway and downstream geraniol synthesis pathway (Fig. 1).

2.2. Plasmids and plasmid construction

E. coli DH5α was used for amplification of all recombinant plasmid constructions. PCR primers and plasmids used in this study are listed in Table 1. *FPPS* (*ispA*) from *E. coli* MG1655 was converted to *GPPS* by site-directed mutation of Ser^{80} into Phe with primers

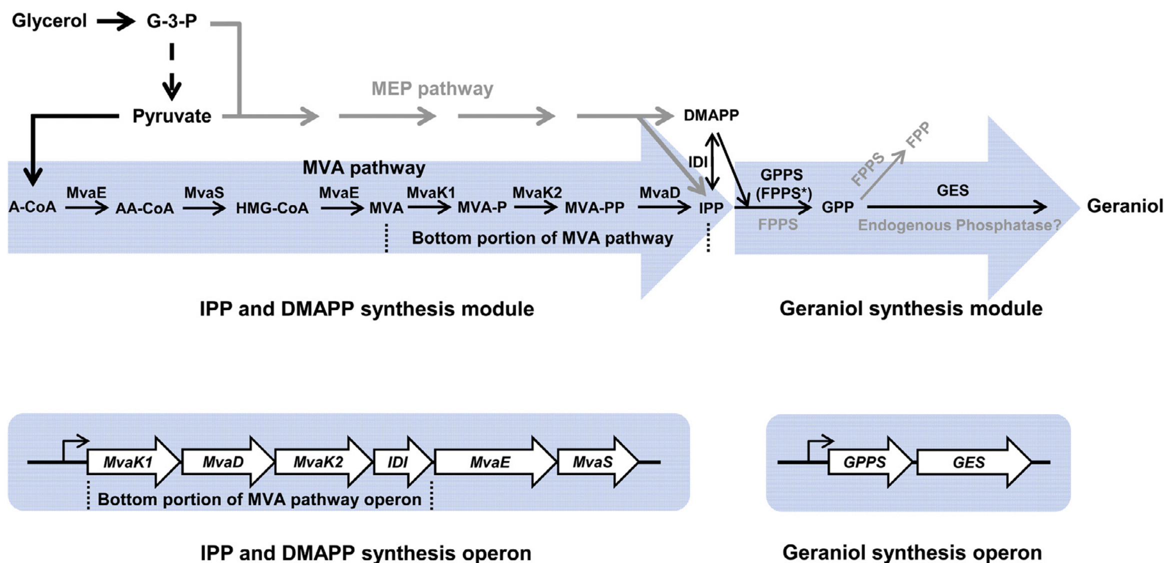

Fig. 1. Modules and corresponding operons of geraniol biosynthesis in engineered *E. coli*. The whole geraniol synthesis pathway is separated into two modules, the upstream IPP/DMAPP synthesis module (left side blue arrow) consisting of an engineered foreign MVA pathway (Yoon et al., 2009) and the downstream geraniol synthesis module (right side blue arrow) consisting of GPP synthase and geraniol synthase. Building blocks of IPP and DMAPP are synthesized from the native MEP pathway (gray arrows) and the foreign MVA pathway using glycerol as the main carbon source. GPP and FPP are synthesized from IPP and DMAPP by GPPS (a mutant derived from FPPS; FPPS*) and FPPS (gray color), respectively. The operons corresponding to the upstream and downstream modules are indicated with solid blue boxes below the pathway diagram. The bottom portion of MVA pathway and its corresponded operon were indicated between two dashed lines, respectively. Open arrows and bent arrows represent genes and promoters, respectively. Abbreviations of the pathway intermediates are as follows: G-3-P, glyceraldehyde-3-phosphate; MEP, methyl-erythritol phosphate; A-CoA, acetyl-CoA; AA-CoA, acetoacetyl-CoA; HMG-CoA, hydroxymethylglutaryl-CoA; MVA, mevalonate; MVA-P, mevalonate 5-phosphate; MVA-PP, mevalonate diphosphate; DMAPP, dimethylallyl diphosphate; IPP, isopentenyl diphosphate; GPP, geranyl diphosphate; FPP, farnesyl diphosphate. Abbreviations of the enzymes are as follows: 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; FPPS, farnesyl diphosphate synthase; GES, geraniol synthase.

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