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Bacterial Production of Pinene by a Laboratory-Evolved Pinene-Synthase

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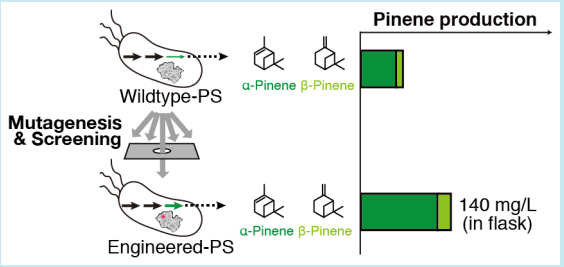
Supporting Information

ABSTRACT: Successful feeding of the substrate geranylpyrophosphate (GPP) to monoterpene synthase is critical to the efficient microbial production of monoterpenes. Overexpression of GPP synthases, metabolic channeling from GPP synthase to terpene synthases, and down-tuning of endogenous competitors have been successfully used to increase the production of monoterpene. Nevertheless, the production of monoterpenes has remained considerably lower than that of hemi-/sesqui-terpenoids. We tested whether it is effective to improve the cellular activity of monoterpene synthases. To this end, we developed a high-throughput screening system to monitor for elevated GPP consumption. Through a single round of mutagenesis and screening, we isolated a pinene synthase variant that outperformed the wild-type (parent) enzyme in multiple contexts in *Escherichia coli* and cyanobacteria. The purified variant exhibited drastically altered metal dependency, enabling to keep the activity in the cytosol that is manganese-deficient. Coexpression of this variant with mevalonate pathway enzymes, isopentenylpyrophosphate isomerase, and GPP synthase yielded 140 mg/L pinene in a flask culture.



KEYWORDS: *terpene synthase, monoterpene bioproduction, carotenoid, directed evolution, cyanobacteria*

Monoterpenes (C_{10}-isoprenoids), include various compounds of great industrial value as flavors, fragrances, and insecticides and more recently as candidate renewable jet fuels and precursors of synthetic polymers.[1,2] To increase the production of terpenoids in general, an increase in the supply of precursors by either overexpression of the endogenous MEP-pathway enzymes or installation of the MEV pathway turned out to be highly effective[3−7] (Figure 1). However, the net effects of these strategies have proven to be limited[3−5,8−14] for monoterpene production. To date, the production level of monoterpenes is over 10-fold lower than that of hemiterpene (isoprene)[15] and sesquiterpenes.[16]

The metabolic pathway for monoterpenes in yeast and *Escherichia coli* (*E. coli*) is unique in that their direct substrate, geranyl pyrophosphate (GPP), is produced only as the intermediate in the two-step synthesis of farnesyl pyrophosphate (FPP) (Figure 1). In these hosts, monoterpene synthases (mono-TPSs) compete for GPP with endogenous FPP synthases (FPPS), which act both as a supplier and highly competitive consumer of GPP. Overexpression of GPP synthases (GPPS) is one of the most popular and effective strategies to increase monoterpene production, but it could also increase the further conversion of GPP into FPP. To reduce this, the chromosomal gene coding FPPS can be replaced with an FPPS with moderately decreased second-step activity,[9,12] achieving the significant improvement in geraniol production. Alternatively, mono-TPSs can be physically linked/fused to GPP synthase to generate substrate channeling between the two enzymes.[11]

The most straightforward approach to increase monoterpene production is to engineer mono-TPSs that can better compete for GPP. The lack of convenient screening systems for TPS activity has been the obstacle to explore this strategy, but there are still several reports in which improvement in cellular activities of sesqui-[17] and di-[18] TPSs resulted in the significant

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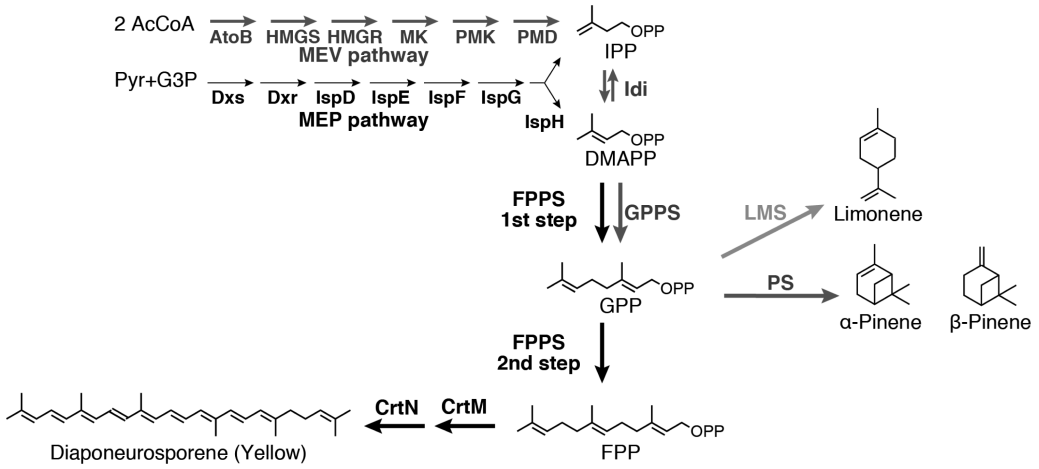

Figure 1. Biosynthetic pathway to pinenes, limonene, and diaponeurosporene. Supplies of IPP and DMAPP could be markedly elevated by overexpressing the rate-limiting enzymes in endogenous (MEP) pathways or by reconstructing other routes (MEV pathways). However, monoTPSs compete for GPP with the second-step activity of the endogenous GPP supplier FPPS. Competitiveness of the PS for GPP could be screened by coexpressing three-enzyme pathways that convert FPP into carotenoid pigments. AcCoA, acetoacetyl-CoA; Pyr, pyruvate; G3P, glyceraldehyde 3-phosphate; IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl pyrophosphate; GPP, geranyl pyrophosphate; FPP, farnesyl pyrophosphate; AtoB, acetoacetyl-CoA synthase; HMGS, HMG-CoA synthase; HMGR, HMG-CoA reductase; MK, mevalonate kinase; PMK, phosphomevalonate kinase; PMD, phosphomevalonate decarboxylase; Idi, isopentenyl pyrophosphate isomerase; Dxs, 1-deoxy-D-xylulose-5-phosphate synthase; Dxr, 1-deoxy-D-xylulose 5-phosphate reductase; GPPS, geranyl pyrophosphate synthase; FPPS, farnesyl pyrophsphate synthase; PS, pinene synthase; LMS, limonene synthase.

elevation in the production level of target terpenes. Recently, we have developed cell-based screening for TPS activity[19] based on the competition of the TPSs with carotenogenic enzymes for their common substrates: higher cellular activities of TPSs correlated with lower levels of precursors available for carotenoid biosynthesis, which is easily detected by reduced pigmentation.

In this work, we attempted to improve the productivity of the synthase of pinene, a valuable monoterpene widely used as the chemical precursor of fragrances and jet fuel.[11] To this end, original colorimetric screening[19] was modified so that the flux from GPP to carotenoid is elevated. A round of mutagenesis and screening yielded a pinene synthase (PS) mutant that appears to better compete for GPP. The expression of the PS mutant resulted in the elevated cellular production level of pinene in various contexts in both *E. coli* and cyanobacteria.

RESULTS AND DISCUSSION

***E. coli* Production of Pinene Using a Wild-Type Pinene Synthase.** A sufficient supply of GPP is key to success in the microbial production of monoterpenes. In *E. coli*, GPP is synthesized by IspA, a two-step enzyme involving GPPS and FPPS. Thus, IspA works as both the supplier of and the competitor for GPP, the substrate for monoterpene synthase. To better compete for GPP in the second-step reaction of IspA (reported *Km* of IspA to GPP is 5.5 μM[20]), we chose (−)-α-pinene synthase from *Pinus taeda*,[21] whose reported *Km* for GPP (1.4 μM) was the lowest among the known PSs. The *P. taeda* PS was codon-optimized for *E. coli*, histidine-tagged, and subcloned into the expression plasmid (pJ404-PS_{his}).

To maximize the supply of precursors (IPP and DMAPP), we constructed the booster plasmid pMEV-idi, based on the plasmid pJBEI-6409[3] developed by Keasling's group. The plasmid pJBEI-6409,[3] expresses all six genes in the MEV pathway, isopentenyl pyrophosphate isomerase (Idi), GPPS, and limonene synthase (LMS), to obtain a high-titer precursor supply. With staphylococcal HMGS and HMGR and codon optimization of most of the genes involved, this plasmid is one of the best available isoprenoid booster plasmids, yielding ~400 mg/L limonene in a flask culture.[3] From this plasmid, we removed the genes encoding GPPS and LMS to obtain pMEV-idi.

Historically, *Abies grandis* GPPS (AgGPPS)[3,4] and the S80F mutant of *E. coli* IspA (IspA(S80F))[5,6,10] have been the two standard GPP suppliers used in microbial production of monoterpenes. We compared the effect of expressing these three enzymes on the *E. coli* production of pinenes. In the absence of overexpression of prenyltransferase, we detected 4.2 mg/L α-pinene (Figure 2). Upon coexpression of *Ag*GPPS or IspA(S80F), the production of pinene reached 50 mg/L. This leap clearly suggests that the conversion of DMAPP to GPP is the major limiting step in the precursor supply. In terms of the booster, we did not observe a significant difference between

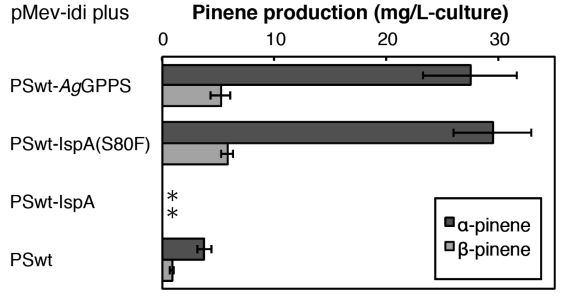

Figure 2. Effect of the coexpression of prenyltransferases on the production of pinene in *E. coli*. Cells containing two plasmids, pMEV-idi and PS-expressing plasmids (pJ404-PS_{his}, pJ404-PS_{his}-*Ag*GPPS, pJ404-PS_{his}-IspA(S80F), and pJ404-PS_{his}-IspA.) were grown in TB medium overlaid with 10% (v/v) dodecane. After 24 h shaking at 30 °C, a fraction of the dodecane was subjected to GC-FID analysis to determine the level of pinene (mg/L of culture). The results are shown as the average of three biological replicates; error bars represent the standard deviation from the mean. Asterisks indicate that no detectable peaks were observed.

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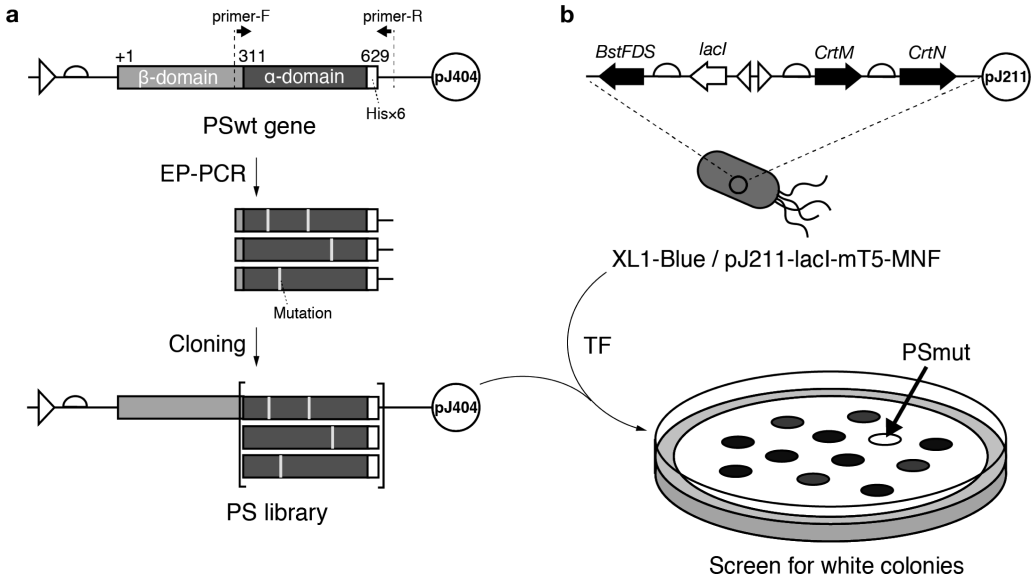

Figure 3. Directed evolution of PS. The catalytic domain (α-domain, residues 311 to 629) with the histidine tag on the C-terminus was subjected to error-prone PCR and subcloned in-frame with the β-domain of PSwt. The resultant plasmid library was transformed into XL1-Blue containing a plasmid expressing carotenoid enzymes and FPPS to identify colonies with lower levels of pigmentation.

AgGPPS and IspA(S80F). Du *et al.*[22] reported that *Ag*GPPS outperformed IspA(S80F) as precursor supplier on LMS-GPPS operons on a middle-copy plasmid, whereas LMS-IspA(S80F) performed better on a high-copy plasmid.

With the coexpression of wild-type-IspA, we observed nearly complete loss of α-pinene production (Figure 2). Overexpression of IspA was also reported to cause a significant reduction in *E,Z*-farnesol production.[23] Indeed, the second-step activity of IspA was reported to be 10 times higher in Vmax/*Km* than the first-step activity.[20] Collectively, a non-negligible fraction of GPP must be wastefully converted to FPP, particularly in cells overexpressing GPPSs. Thus, we aimed to find PS mutants that better compete for GPP with the second step activity of endogenous IspA.

Screening for Mono-TPS Activity. Previously, we reported colorimetric screening for mono-, sesqui-, and di-TPS activity based on competition for substrates with carotenoid-synthesis pathways.[19] In that work, the C_{30} carotenoid pathway was used as a probe to preferentially visualize the cellular activities of FPP consumers (sesqui-/triterpenoid synthases), whereas the lycopene pathway was used for the selective visualization of GGPP consumer (diterpene synthase) activity. The activity of mono-TPSs also decreased the pigmentation of cells containing the C_{30} carotenoid pathway (CrtM/CrtN).[19] This finding suggests that PS significantly reduces the availability of FPP to the C_{30} carotenoid pathway by partially intercepting the precursor GPP. Indeed, the transformation of PS resulted in a marked reduction in colony pigmentation of XL1-Blue containing pJ211-lacI-mP$_{T5}$-MN. It was easy to distinguish functional PS (wild-type; PSwt) from nonfunctional PS (PS(D380A)) (Supporting Information, Figure S1). However, cells transformed with PSwt formed colonies with a faint color, and we expected difficulty in discriminating the colonies with further reduced pigmentation. A higher screening threshold for PS mutant activity was needed to identify PS variants with higher cellular activity.

To this end, we decided to plasmid-express prenyltransferase to moderately elevate the GPP-to-FPP activity in the cell. Because overexpression of IspA resulted in the nearly complete failure of pinene synthesis (Figure 2), we decided to express FPPS from *Geobacillus stearothermophilus* (BstFPPS), a thermophilic ortholog of IspA in turn, with moderate promoter. Probably with better channeling from GPP to FPP, the pigmentation level (colony hue) of the cell became higher under all conditions. Most importantly, the cells formed bright yellow colonies, even with the coexpression of PSwt (Figure S1). We concluded this construct is suitable for the isolation of PS variants with elevated GPP-consumer activity.

Screening for PS Mutants with Higher Cellular Activity. Theoretically, cellular activity of enzymes could be elevated by two different mechanisms: improvement in the catalytic properties or improvement in the effective concentration of enzymes. The latter could be achieved by improving either the enzyme stability or the transcription or translation efficiency. To avoid isolating the mutants with improved translation-initiation efficiency, we excluded the first 35 nucleotides of the reading frame of PS, which strongly affect translation efficiency,[24] from the mutagenesis. Additionally, we decided to introduce random nucleotide mutations only in the catalytic domain (α-domain, residues 311−629) by error-prone PCR (Figure 3a). The resultant PS library was introduced into XL1-Blue harboring pJ211-lacI-mP$_{T5}$-MNF and plated onto agar topped with nitrocellulose membranes for colorimetric screening (Figure 3b). Approximately 25% of the transformants showed intense yellow pigmentation. We classified these transformants as "dead mutants" as they likely contained deleterious mutations, including nonsense, frame-shift, and/or insolublizing mutations. The majority (~75%) of the transformant pool yielded slightly yellow colonies that were indistinguishable from the cells containing PSwt. The screening threshold was adjusted to isolate PS mutants with higher activity, and PSwt could not completely abolish the yellow pigmentation.

In the first screening plate containing 370 colonies, there were three colonies with significantly weaker pigmentation than others. We isolated their plasmids and introduced them into fresh cells containing carotenoid plasmid (pJ211-lacI-mP$_{T5}$-

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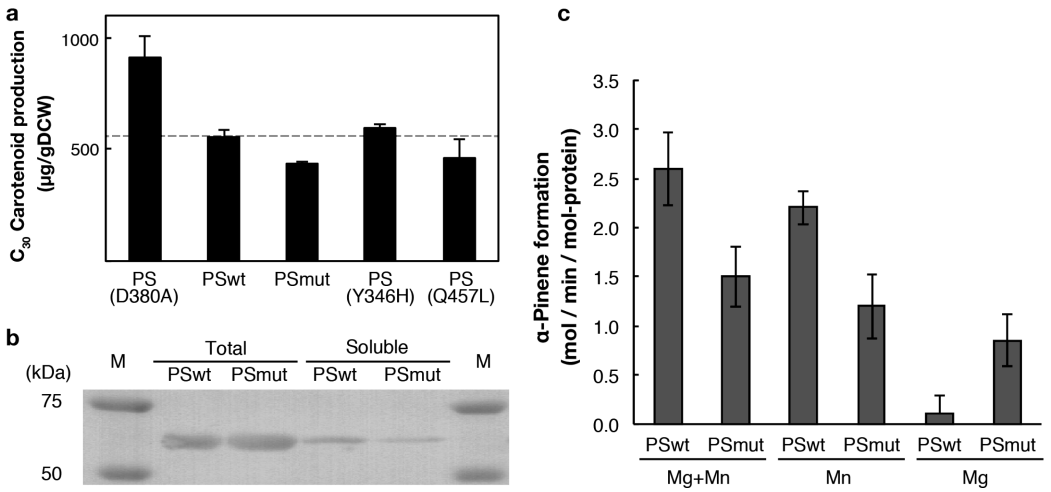

Figure 4. Characterization of the PSwt and its variants. (a) Performance of the C_{30} carotenoid biosynthetic pathway in the presence of PS mutants coexpressed in *E. coli* (XL1-Blue). Carotenoid producing plasmid pJ211-lacI-mP$_{T5}$-MNFI was transfected into the cell containing the plasmid expressing either of the PS mutants. After 48 h of shaking in TB media, the carotenoids accumulated in each cell were extracted with acetone to determine the level of pigment production. (b) Western blot analysis of the total/soluble fraction of the XL1-Blue expressing PSwt and PSmut. (c) Analysis of the cofactor preference of PSwt and PSmut. Purified PSwt and PSmut were incubated with GPP in the buffer containing $MgCl_2$, $MnCl_2$, or both at 25 °C, and the product (α-pinene) formation was tracked using GC-FID. Data shown in panels a and c are the average of three independent experiments with error bars representing the standard deviation from the mean.

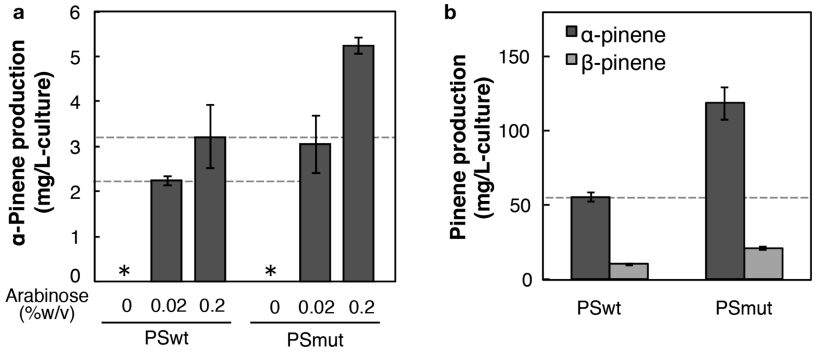

Figure 5. Pinene production of PS mutant and wild-type bacteria. Cells were transformed with pJ404-PSs and pBBRSOE6 (a) or pMEV-idi-GPPS (b). The cells containing plasmids were grown in TB (containing 4% glycerol as a carbon source) medium with 10% dodecane overlay at 30 °C with 50 μM IPTG in a 200 mL flask. Pinene production was measured at 24 h. Results are shown as the average of three biological replicates, with error bars representing the standard deviation from the mean.

MNF). Two of the three exhibited wild-type-like phenotypes (false positives). However, one of the three transformants showed significant pigment reduction and reduced pigment accumulation in liquid culture (PSmut, Figure 4a).

Characterization of the PS Mutant. Sequence analysis (Figure S2) identified two amino-acid substitutions (H346Y and Q456L) in the α-domain (catalytic domain) of PSmut. To determine which mutation contributes to elevated cellular activity, we created single mutants of PS (H346Y) and PS (Q457L) by site-directed PCR mutagenesis. When coexpressed with a carotenoid pathway, PS (Q457L) showed a greater reduction in pigmentation, whereas PS (H346Y) was indistinguishable from PSwt (Figure 4a). Thus, we concluded that Q467L, not H346Y, was responsible for the phenotype change. Q457 is located on helix F, which supports a part of the catalytic core and is proximate to the highly conserved residues Y455 and E458, which correspond to Y426 and E429 of bornyl pyrophosphate synthase.[25] These two residues were shown to contact pyrophosphate and the cofactor ion through water molecules.[25]

Directed evolution for cellular activity frequently yields "expression mutants" with higher effective enzyme concentrations from increased protein stability, a prolonged half-life of mRNAs, removal of rare codons, or modification of the translational initiation efficiency. However, Western blot analysis revealed no significant differences between mutants and PSwt in the total and soluble fractions (Figure 4b). This result indicates that the greater reduction of carotenoids is not caused by elevated PS concentration, and presumably by the elevation in enzyme activity of PS.

To examine how PSmut performs better than PSwt, purified PSs were compared in activity in various buffer conditions (Figure 4c). Owing to the known variation in metal-dependency, *in vitro* analysis of monoterpene synthases have been typically conducted in buffer containing both of $MgCl_2$ and $MnCl_2$.[21] In the presence of Mg^{2+} and Mn^{2+}, the activity of PSmut was rather lower than PSwt. However, the PSmut exhibited about 7 times higher activity than PSwt in the reaction condition free from Mn^{2+} (buffer supplemented only with $MgCl_2$, Figure 4c). Typically, conifer monoterpene cyclases require Mn^{2+} as a cofactor, and Mg^{2+} cannot substitute

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it.[26] The known optimal concentration of Mn^{2+} (>0.5 mM) for monoterpene synthases[26,27] is much higher than the cytosolic concentration of manganese in *E. coli* (*ca.* 10 μM).[28] In good agreement with this, the activity of PSwt in the absence of Mn^{2+} was only ca. 1/25−1/20 of that in Mn^{2+}-containing conditions. In contrast, PSmut remained 60% active in the buffer supplemented only by Mg^{2+}. This suggests that Q457L alters the cofactor preference of PS, thereby enabling PS to stay highly active upon binding with magnesium, which is 3 orders of magnitude higher (10−20 mM)[28] in cytosol. Concentrations of manganese were reported to be equal or even higher than those of magnesium[29−31] in Pinaceae needles, from which the cDNA of *P. taeda* PS was isolated.[21]

***E. coli* Production of Pinene Using PS Mutants.** The performance of PSmut and PSwt were compared in *E. coli* in various contexts. First, PS-expressing plasmids were introduced to *E. coli* with a reinforced MEP pathway. To this end, we transfected *E. coli* with pBBRSOE6,[6] the plasmid expressing Dxs, Idi, and IspA(S80F), under the P_{BAD} promoter (Figure 5a). The expression of Dxs and Idi elevates the level of DMAPP and IPP in the cell, and the expression of IspA (S80F) increases the cellular availability of GPP. With the induction of these enzymes by L-arabinose, XL1-Blue containing PSwt produced ∼3 mg/L pinene. When transformed with PSmut with the same construct and under the same culture conditions, the cell accumulated ∼5 mg/L pinene (60% improvement).

By switching the MEP booster to an MEV booster, Martin *et al.*[7] improved sesquiterpene production by 10-fold. To determine whether similar results could be observed for monoterpenes, we constructed pMEV-idi-GPPS by removing the gene encoding LMS from pJBEI-6409.[3] In this context, the cells expressing PSmut accumulated twice as much pinene as those expressing PSwt (Figure 5b), producing 140 mg/L pinene (120 mg/L α-pinene and 20 mg/L β-pinene) in 24 h. This accumulation is 4.4-fold higher than the highest reported value (32 mg/L[11]) under similar conditions (shaking flask without continuous feeding of carbon source).

The superiority of PSmut over PSwt was further confirmed in two additional contexts. First, the PS-expressing plasmids were introduced into cells containing pJBEI-6409. In this context, PS competes for the precursor with another monoTPS, limonene synthase (LMS) (Figure 6). The production of pinene was significantly reduced (compared with Figure 5b) due to the competition with LMS for GPP. However, the level of PSmut (40 mg/L) was twice as high as it was in cells expressing PSwt (20 mg/L) under these conditions.

We also constructed an in-frame fusion of *Ag*GPPS and PSs (PS-GPPS) (Figure S3). The fusion of TPS and GPPS improved production in some cases, likely *via* channeling effects.[11] Here again, PSmut was superior to PSwt: expression of PSwt-GPPS yielded 70 mg/L α-pinene and 10 mg/L β-pinene, whereas PSmut-GPPS yielded 130 mg/L α-pinene and 20 mg/L β-pinene, achieving the highest product level (150 mg/L in total) in this work. Interestingly, however, fusion of PSs to GPPS at the N-terminus radically reduced the performance of PSs, whereas C-terminal fusion of GPPS did not have a negative impact on the apparent activity of PS (Figure S3). This finding contradicts the previous report for PSs, in which they observed loss of PS activity on fusion to GPPS at the C-terminus, whereas it showed comparable or higher cellular performance in fusion to GPPS at the N-terminus.[11]

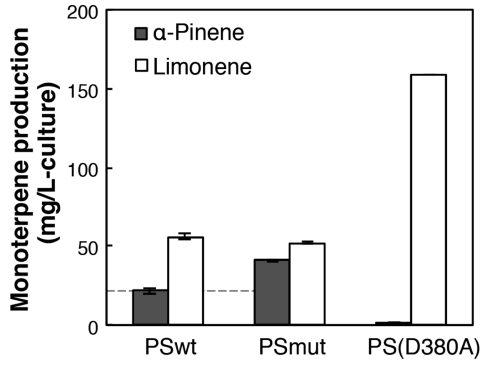

Figure 6. Production of monoterpenes by *E. coli*-expressing PSs and LMS. XL1-Blue were transformed with pJ404-PSs and pJBEI-6409 grown in TB medium containing 4% glycerol as a carbon source, overlaid with 10 v/v% dodecane. After shaking for 24 h at 30 °C with 50 μM IPTG in a 200 mL flask, the levels of pinenes and limonene were determined by GC-FID. Results are shown as the average of three biological replicates with error bars representing the standard deviation from the mean.

Cyanobacterial Pinene Production Using PS Variants. Cyanobacteria have attracted considerable attention as potential production systems for terpenoids due to their capacity to directly convert CO_2 into hydrocarbons.[32] We also tested and compared the performance of PSmut and PSwt in *Synechocystis* sp. PCC 6803.

PSs were introduced into a silent locus on the *Synechocystis* chromosome using PS-recombination plasmids derived from pT31CTH-LMS.[33] The resulting strains were grown under photosynthetic conditions. The time course of pinene accumulation in the cold trap was tracked (Figure 7). The

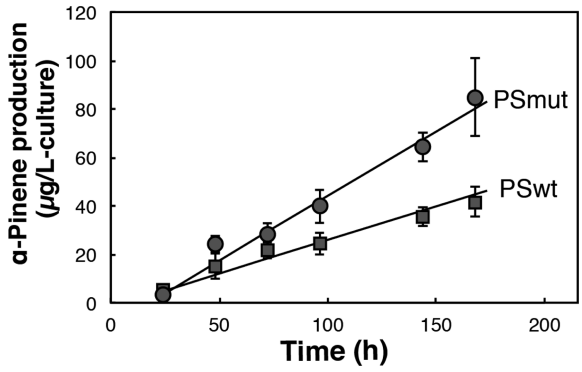

Figure 7. Cyanobacterial production of pinene. The results shown are the average of three biological replicates with error bars representing the standard errors.

cells expressing PSwt accumulated ∼40 μg/L pinene in 168 h, whereas the cells expressing PSmut accumulated twice as much pinene (∼80 μg/L) under the same condition. Thus, PSmut outperformed the wild type in cyanobacteria as well.

The amount of pinene accumulated was approximately one third of the reported value (250 μg/L) for limonene synthesis by LMS-expressing *Synechocystis* sp. PCC 6803 using a nearly identical expression construct and downstream process.[33] This yield likely reflects the higher catalytic capacity of LMS over PS. Indeed, the amount of limonene was always higher than that of pinenes when we coexpressed LMS and PSs in the same *E. coli* cell (Figure 6).

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Table 1. Plasmids Used in This Study

name	marker/ori	construct notes	ref
pJ404-PS$_{his}$	Amp^R/pUC	P_{lacI}-*lacI*, $P_{T5:lacO}$-*PS*	this work
pJ404-PS$_{his}$-GPPS	Amp^R/pUC	P_{lacI}-*lacI*, $P_{T5:lacO}$-*PS-AgGPPS*	this work
pJ404-PS$_{his}$-ispA(S80F)	Amp^R/pUC	P_{lacI}-*lacI*, $P_{T5:lacO}$-*PS-ispA(S80F)*	this work
pJ404-PS$_{his}$-ispA	Amp^R/pUC	P_{lacI}-*lacI*, $P_{T5:lacO}$-*PS, ispA*	this work
pJ404-PSmut$_{his}$	Amp^R/pUC	P_{lacI}-*lacI*, $P_{T5:lacO}$-*PSmut*	this work
pJ404-PS(D380A)$_{his}$	Amp^R/pUC	P_{lacI}-*lacI*, $P_{T5:lacO}$-*PS(D380A)*	this work
pJ404-PS(Q457L)$_{his}$	Amp^R/pUC	P_{lacI}-*lacI*, $P_{T5:lacO}$-*PS(Q457L)*	this work
pJ404-PS(H346Y)$_{his}$	Amp^R/pUC	P_{lacI}-*lacI*, $P_{T5:lacO}$-*PS(H346Y)*	this work
pET15b-PS$_{his}$	Amp^R/pUC	P_{lacI}-*lacI*, $P_{T7:lacO}$-*PS*	this work
pET15b -PSmut$_{his}$	Amp^R/pUC	P_{lacI}-*lacI*, $P_{T7:lacO}$-*PS (H346Y, Q457L)*	this work
pT13CTH-PSwt	Amp^R/pUC	*Ptrc-hisPS*	this work
pT13CTH-PSmut	Amp^R/pUC	*Ptrc-hisPSmut*	this work
pJBEI-6409[a]	Cm^R/p15A	P_{lacI}-*lacI*, $P_{lacUV5:lacO}$-*atoB-hmgs-hmgr*, $P_{Trc:lacO}$-*MK-PMK-PMD-idi*, $P_{Trc:lacO}$-AgGPPS, *LMS*	Alonso-Gutierrez *et al.*, 2013
pMEV-idi	Cm^R/p15A	P_{lacI}-*lacI*, $P_{lacUV5:lacO}$-*atoB-hmgs-hmgr*, $P_{Trc:lacO}$-*MK-PMK-PMD-idi*	this work
pMEV-idi-GPPS	Cm^R/p15A	P_{lacI}-*lacI*, $P_{lacUV5:lacO}$-*atoB-hmgs-hmgr*, $P_{Trc:lacO}$-*MK-PMK-PMD-idi*, $P_{Trc:lacO}$-AgGPPS	this work
pBBRSOE6[b]	Kan^R/pBBR1	P_C-*araC*, P_{BAD}-*dxs*-Hp*idi-ispA*(S80F)	Reiling *et al.*, 2004
pJ211-lacI-mP$_{T5}$-MN[c]		P_{lacI}-*lacI*-Bst*fpps*, mP$_{T5:lacO}$-*crtM-crtN*	
pJ211-lacI- mP$_{T5}$-MNF[iii]	Kan^R/p15A	P_{lacI}-*lacI*-Bst*fpps*, mP$_{T5:lacO}$-*crtM-crtN*	this work
pJ211-lacI- mP$_{T5}$-MNFI	Kan^R/p15A	P_{lacI}-*lacI*-Bst*fpps*, mP$_{T5:lacO}$-*crtM-crtN*	this work

[a]Acetoacetyl-CoA synthase from *E. coli* (*atoB*), HMG-CoA synthase (*hmgs*), HMG-CoA reductase (*hmgr*) from *Staphylococcus aureus*, mevalonate kinase (*MK*), phosphomevalonate kinase (*PMK*), phosphomevalonate decarboxylase from *S. cerevisiae* (*PMD*), isopentenyl pyrophosphate isomerase from *E. coli* (*idi*), geranyl pyrophosphate synthase (Ag*GPPS*) from *Abies grandis*, limonene synthase (*LMS*) from *Mentha spicata*. [b]1-Deoxy-D-xylulose-5-phosphate synthase (dxs) from *E. coli*, isopentenyl pyrophosphate isomerase from *Haematococcus pluvialis* (Hp*idi*), farnesyl pyrophsphate synthase from *E. coli* contains S80F mutation (*IspA*(S80F)). 'Farnesyl pyrophsphate synthase from *Geobacillus stearothermophilus* (Bst*fpps*), *crtM* and *crtN* from *Staphylococcus aureus*.

CONCLUSIONS

TPSs have been identified as the major limiting factor in the production of terpenoids, particularly in the context of high-flux conditions.[7,34] To improve the activity of TPSs, semirational designs of folding stability[17] and carbocation intermediates[18] and laboratory evolution for stability using CAT-based screening[35] were attempted for several TPSs. The use of engineered TPS indeed increased the yield from the microbial production of some TPSs.[17,18]

To significantly improve the performance of the enzymes, directed evolution is the most preferable and proven approach. To enable the directed evolution of TPSs with various product types, high-throughput screening has been designed mainly by the visualization of substrate consumption. Pyrophosphates released by TPSs can be colorimetrically detected by selective chelating agents[36] or by coupling with oxidative colorimetric reactions.[37] Alternatively, depending on the product type, activity of TPSs in cell lysate can be visualized using surrogate substrates designed to release methanol upon cyclization.[38] However, all these systems require purification of enzymes or at least preparation of the lysate. Additionally, cellular activity could be highly context-dependent, and therefore, the activity (or performance) of TPS should be screened in every condition of cellular production.

The first cell-based, and thus high-throughput, selection for TPS activity was reported by Withers *et al.*,[39] who took advantage of the toxicity of overaccumulated prenylpyrophosphates. Plasmid-based expression of its consumer (TPSs) decreases the level of prenylpyrophosphate, thereby restoring the cell growth. Our screening method is based on the decrease in carotenoid pigments due to the redirection of the prenylpyrophosphate molecules to TPSs.[19] However, we had to modify this method for robust use in the directed evolution of mono-TPS because mono-TPSs directly compete for GPP with FPPS, not carotenoid synthetic enzymes. Through the addition of FPPS to the screening plasmid, followed by the careful expression tuning, we successfully established a screening system suitable for the detection of mono-TPS mutants with improved cellular performance. By tuning the screening constructs, additional rounds of directed evolution could be performed.

We obtained a mutant of PS (PSmut) in a single round of mutagenesis/screening. As far as we know, this is the first example of improving activity of monoterpene synthase. This improvement was ascribed to the change in cofactor preference, thereby enabling the use of magnesium that is 3 orders of magnitude higher in concentration in the cell.[28] Thus, Mn^{2+}-limiting nature of the cytosol could be a previously overlooked obstacle to achieve hyperproduction of monoterpenes.

Finally, PSmut out-performed PSwt in all tested *in vivo* contexts, differing in titer by 3 orders of magnitude (100 μg/L/168 h in cyanobacteria (Figure 7) to 140 mg/L/24 h in *E. coli* (Figure 5b)). The highest production efficiency obtained (140 mg/L/24 h in flask culture) (Figure 5b) was approximately four times the highest reported value.[11] To our knowledge, this is the first report of increasing monoterpene productivity by the directed evolution of monoterpene synthases.

MATERIALS AND METHODS

Strain and Plasmids. *E. coli* XL1-Blue strain (*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [*F′ proAB lacIqZDM15 Tn10 (Tetr)*]) (Stratagene, La Jolla, CA,) was used for DNA cloning, colorimeric screening, and bioproduction of monoterpenes. *E. coli* BL21(AI) strain (Life Technologies, Carlsbad, CA) was used for PS purification.

pMev-idi and pMEV-idi-GPPS were derived from pJBEI-6409,[3] which was obtained from Addgene. pJBEI-6409 originally encodes six genes in mevalonate pathways (Mev: *atoB* from *E. coli*, *HMGS* and *HMGR* from *Staphylococcus aureus* and *MK*, *PMK*, and *PMD* from yeast), isopentenyl

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pyrophosphate isomerase (*idi*) from *E. coli*, and GPP synthase (*GPPS*) from *Abies grandis*, limonene synthase (LMS) from *Mentha spicata*. The parts of the synthetic operon (either Mev-idi or Mev-idi-GPPS) were PCR-amplified and were subcloned using SLIC[40] into the *Sma*I/*Xho*I site of pJBEI-6409, resulting in pMev-idi and pMEV-idi-GPPS.

pJ211-lacI-mP$_{T5}$-MNF and pJ211-lacI-mP$_{T5}$-MNFI were constructed as follows: genes coding for CrtM and CrtN from *S. aureus* (codon-optimized for *E. coli*) were synthesized and subcloned into the pJ211 cloning vector by DNA 2.0 Inc. (Menlo Park, CA). The synthetic T5/lacO promoter (mT5P: −35 resion of T5 promoter was modified from TTGCTT to GTGCAA) was inserted using the FASTR method to regulate gene expression of CrtM and CrtN. *Geobacillus stearothermophilus fpps* and *E. coli idi* (PCR-amplified from pUC-fds and pAC-idi,[19] respectively) were inserted downstream of P_{lacI} by SLIC, yielding pJ211-lacI-mP$_{T5}$-MNF and pJ211-lacI-mP$_{T5}$-MNFI.

Plasmid pJ404-PS$_{his}$ was constructed as follows: *Pt1*, the gene coding (−)-α-pinene synthase from *Pinus taeda*,[21] was truncated by 65 aa from its N-terminus and codon-optimized for *E. coli*, and a 6×His tag was added to its C-terminus. The designed sequence (named PS in this work), synthesized by DNA 2.0 Inc., was subcloned into the vector pJ404.

pJ404-PS-GPPS, pJ404-PS-ispA(S80F), and pJ404-PS-ispA were constructed by inserting Ag*GPPS* from pJBEI-6409, *ispA*(*S80F*) from pBBRSOE6, and *ispA*, with synthetic RBSs (RBS scores[24] for *ispA*(30898) and Ag*GPPS* (20607), respectively). pJ404-PS(D380A)$_{his}$ was constructed by replacing the Asp 380 codon (GAT) of *PS* on pJ404-PS$_{his}$ with the Ala codon (GCG). To create this mutation, we conducted SLIC cloning with two PCR fragments (upstream part of *PS*, ampified with PS-ADMYD-R and PS-Chis-F (see Table S1 for the primers used); the downstream part of *PS*, amplified with PS-ADMYD-F and PS-Chis-R) were assembled with *NdeI*/*XhoI*-treated pJ404. Another plasmid expressing PS variants (pJ404-PS(Q457L)$_{his}$, pJ404-PS(H346Y)$_{his}$, and pJ404-PS-(H346Y/Q457L)$_{his}$) was constructed by Golden Gate cloning[41] using the primer pairs PSH346Y-F and PSH346Y-R and/or PSQ457L-F and PSQ457L-R.

Construction of the PS-Variant Library. Random mutations were introduced into the α-domain of the PS gene (residues 311 to 629) through error-prone PCR[42] (with Taq polymerase (New England Biolabs, Hitchin, Herts, UK) and 10 μM $MnCl_2$ in Thermopol Buffer, ~ 1000-fold amplification and 25 effective cycles). The resulting PCR fragments were digested with *Hin*dIII and *XhoI* and ligated with a pJ404 vector containing the β-domain of the PS gene using T4DNA ligase (New England Biolabs). A portion of the ligation product was transformed into chemically competent XL1-Blue cells prepared with the *E. coli* Transformation Buffer Set (Zymo Research Corporation, Irvine, CA). After culture in SOB medium for 1 h (37 °C), the transformed cells were shaken overnight in 40 mL of fresh LB culture. An aliquot of the culture was miniprepped to obtain a plasmid library pJ404-[PS]$_{his}$. Alongside, a portion (1/50) of this culture was plated onto LB medium. The effective library size (number of unique mutants in the library) was determined to be 16 000 from the number of colonies on the plate.

Screening for PS Variants with Elevated Substrate Consumption. *First Screening.* pJ404-[*PS*]$_{his}$ was introduced into chemically competent XL1-Blue cells containing pJ211-lacI-mP$_{T5}$-MNF. The transformants were plated onto LB agar (carb/kan) topped with a nitrocellulose membrane (Pall corporation, Port Washington, NY). After incubation at 37 °C for 20 h, the nitrocellulose membrane was carefully lifted and transferred onto an LB agar (carb/kan) plate containing 100 μM IPTG. The plates were left at room temperature for another 24−48 h in darkness or dim light. The plate was scanned using a desktop flatbed scanner. From the original color image, the blue channel was extracted to score the pigmentation of each colony. The colonies that were significantly weaker in color were subjected to overnight culture in isolation, from which plasmid DNA was extracted. The plasmid isolated was reintroduced into XL1-Blue containing pJ211-lacI-mP$_{T5}$-MNF and grown on LB agar to confirm the phenotype (reduction in pigmentation).

Second Screening. The plasmid isolated was introduced into XL1-Blue containing pJ211-lacI-mP$_{T5}$-MNFI. The transformants cells were inoculated into 500 μL of LB medium in a deep 96-well plate and cultured at 37 °C and 1,000 rpm for 16 h (preculture). A 20 μL aliquot of the preculture was transferred to 2 mL of fresh TB (carb/kan) in a deep 48-well plate and cultured at 30 °C and 1000 rpm for 8 h. Then, 100 μM IPTG was added to each of the wells, and the plates were shaken for 16 h. The cells were individually harvested, washed with saline, and centrifuged to obtain cell pellets. After the supernatants were discarded and the cell pellets were briefly vortexed, 1 mL of acetone was added to each of the pellets. The pellets were immediately vortexed for 1 min to extract the carotenoids and then centrifuged. The absorbance spectra (400−600 nm at 5 nm intervals) were analyzed for acetone extracts using a SpectraMax Plus Absorbance Microplate Reader (Molecular Devices, Sunnyvale, CA). The peak heights of the resulting extracts at 470 nm were used to score the pigmentation level of each culture using the molar adsorption coefficients of diaponeurosporene (470 nm, 147 000 M^{-1} cm^{-1}).

Determination of the Production Level of Pinene. XL1-Blue was transformed with PS plasmids (pJ404-PS$_{his}$ or pJ404-PSmut$_{his}$) and GPP-carrying plasmids (pBBRSOE6, pMEV-idi-GPPS, or pJBEI-6409). The transformants were picked and inoculated into 2 mL of LB medium in a deep 48-well plate and cultured at 37 °C and 1000 rpm, for 16 h. A 500 μL aliquot of these precultures was transferred to 20 mL of TB (4% glycerol) in a flask and cultured at 30 °C and 200 rpm.

Each culture was subjected to induction upon adding 50 μM IPTG when the cell density reached an OD_{600} of ~0.4−0.7. Immediately after the induction, we overlaid 2 mL (10 v/v% of the original culture) of dodecane onto the cultures and shook them for an additional 24 h at 30 °C. Then, 5 μL of dodecane fraction was collected and diluted in 495 mL of ethyl acetate spiked with limonene as an internal standard. Thus, obtained samples were analyzed by GC-FID (Shimadzu GC-2014, Shimadzu, Kyoto, Japan) with an Rtx5 ms capillary column (30 m × 25 mm ID and 0.25 mm film thickness, Restek Corporation, Bellefonte, PA, USA). Splitless injections (1 μL) were performed with an injector and an FID detector temperature of 250 °C and separated with a GC oven-temperature program starting at 60 °C for 2 min, which was increased by 6 °C min^{-1} up to 150 °C and then increased by 20 °C min^{-1} until it reached 230 °C. For quantification, the calibration curve was drawn with a pinene standard (TCI, Tokyo, Japan).

Western Blot Analysis. The cells expressing PSs (XL1-Blue transformed with pJ404-PS$_{his}$ or pJ404-PSmut$_{his}$) were cultured in TB (carb, 4% glycerol) until the OD_{600} reached *ca.*

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0.6. The cells were then induced with 50 μM IPTG and subjected to shaking incubation for an additional 16 h. The cells ($\sim 10^9$ cells) were pelleted and lysed using 100 μL of B-PER reagent (Thermo scientific, Waltham, MA) containing lysozme (Sigma-Ardrich, St. Louis, MO) and DNase (Thermo scientific, Waltham, MA). Next, 20 μL of the resultant lysate was taken as the total fraction. The remainder of each sample ($\sim$80 μL) was centrifuged at 16 900 rcf for 30 min, and 20 μL of its supernatant was taken as the soluble fraction. After dilution in 2× volume of sample buffer (glycerol, SDS, stacking gel buffer, BPB, 2-Mercapt ethanol), 2 μL of the total fraction and 8 μL of the soluble fraction were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis.

The proteins were transferred from the polyacrylamide gel to polyvinylidene difluoride membranes (Immobilon-FL Membrane, 0.45-μm pore size, Millipore, Billerica, MA) using the semidry blotting transfer system (Bio Craft, Tokyo, Japan). The membrane was blocked with 5% skim milk in TBS containing Tween20 (TBST) for 1 h and then washed and incubated with HRP-conjugated 6×his antibody (6×His mAb-HRP Conjugate, Clontech, Mountain View, CA) in TBST for 1 h at room temperature. Finally, the membranes were washed with TBST and stained with diaminobenzidine.

Purification of PSs. A modified pET15b vector (from pET-*fds*[43]) was amplified with primers pET15b-F-XhoI and pET15b-R-BamHI. The DNA fragment was digested with *Xba*I/*Xho*I (vector strand). pJ404-PtPS$_{his}$ and pJ404-PtPSmut$_{his}$ were also digested with *Xba*I/*Xho*I and ligate with the vector strand to generate pET15b-PSwt$_{his}$ and pET15b-PSmut$_{his}$, respectively (PS-overexpressing plasmids).

BL21(AI) was transformed with pET15b-PSwt$_{his}$ or pET15b-PSmut$_{his}$. The transformants were picked and inoculated into 2 mL of LB medium in a deep 48-well plate and cultured at 37 °C and 1000 rpm, for 13 h. A 400 μL aliquot of these precultures was transferred to 40 mL of TB (2% glycerol) in a flask. Each culture was shaken at 37 °C, 200 rpm, and spiked with 100 μM IPTG and 0.2%(w/v) L-arabinose when the OD_{600} reached 0.6−0.8. Each culture was then shaken at 20 °C for an additional 20 h before harvest.

Cells harvested from 20 mL of each culture were suspended in 5 mL of 100 mM Tris-HCl at pH 7.0, 250 mM NaCl, 0.1 mM PMSF, and 10% glycerol and lysed on ice by sonication. PS variants were purified from the lysate using a His SpinTrap column and desalted using a PD MiniTrap G-25 column (both from GE Healthcare), following the manufacturer's instructions. The buffers used were binding buffer (100 mM Tris-HCl pH 7.0, 250 mM NaCl, 10% glycerol, 25 mM imidazole), elution buffer (100 mM Tris-HCl pH 7.0, 250 mM NaCl, 10% glycerol, 100 mM imidazole) and desalting/storage buffer (100 mM Tris-HCl pH 7.0 and 10% glycerol). The concentrations of the resultant proteins were measured by BCA assay (Pierce BCA Protein Assay Kit, Thermo scientific, Waltham, MA). The purified proteins were stored at −20 °C after adding 1 mM DTT.

***In Vitro* Reaction of PS Variants.** The assay was performed as described previously[44] with slight modifications. Each assay mixture contained, in a final volume of 400 μL, 500 mM KCl, 1 mM DTT, 100 mM Tris-HCl pH 7.0 and 10% glycerol, typically 10 nM of purified PS variant, 10 μM GPP (Sigma-Ardrich, St. Louis, MO) as a substrate, and divalent metal salts (either of $MnCl_2$ (5 mM), $MgCl_2$ (5 mM), or both). To trap the monoterpene products on the course of reaction, 200 μL of hexane, spiked with limonene as an internal standard, was overlaid. The reaction mixtures were incubated at 25 °C in 20 min and vortexed to stop the reactions. The hexane fraction was collected and analyzed by GC-FID as described above.

Construction of the PS-Expressing Strains of Cyanobacteria. pT31CTH-PSwt and pT31CTH-PSmut were derived from pT31CTH-LMS, which contains a trc promoter, 6×His-tagged LMS,[33] and a chloramphenicol-resistant cassette, by replacing the LMS gene with PS variants. The resultant plasmid DNA was introduced by double homologous recombination into the *Synechocystis* chromosome, yielding transformants in which part of a silent *slr2031* gene was replaced.[45] Because cyanobacterial cells possess multiple copies of identical chromosomes,[46] complete segregation of the integrated genes was confirmed by PCR and sequencing.

Culturing, Gas-Stripping, and GC Analysis for the Quantification of Pinene Production. Recombinant cells were maintained in the presence of 20 μg/mL chloramphenicol but cultivated in the absence of any antibiotics for pinene production. Growing cells were collected by centrifugation and resuspended in a fresh BG11 medium at a density of 2×10^8 cells/mL. The cultures were bubbled with 1% (v/v) CO_2 under continuous illumination with white fluorescent lamps at 20 μmol photons s^{-1} m^{-2}. The culture flask and cold trap equipped with a Dimroth condenser at −15 °C were connected by a polytetrafluoroethylene tube to recover off-gases into the octane phase. Resulting octane fractions were analyzed using QP2010 Plus GC−MS (Shimadzu, Kyoto, Japan) equipped with a ZB-AAA column (Phenomenex, Torrance, CA). The analytical conditions were as previously described. The pinene peak was identified as the retention time in the selected ion chromatogram. In the quantitative analysis, we used limonene as an internal standard.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.6b00140.

The colony hue (pigmentation) of PS-expressing *E. coli* strains harboring pJ211-lacI-mT5-MN and pJ211-lacI-mT5-MNF; the structural mapping mutations found in PSmut; the pinene production of *E. coli* harboring fusions of PS and GPPS; the sequences of pJ211-lacI-mP$_{T5}$-MNF, and pJ404-PS$_{his}$, and the primer sequences for the experiments (PDF)

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Author Contributions

M.T. and D.U. were involved in the experimental design. M.T. performed experiments using *E. coli*. M.T. and Y.I. conducted purification and *in vitro* analysis of PS mutants. H.K. performed the experiments of cyanobacteria. M.T., S.K.-N., and D.U. analyzed the data. M.I., K.S., and D.U. supervised the project. M.T. and D.U. wrote the manuscript.

Notes

The authors declare no competing financial interest.

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