Applied Microbiology and Biotechnology
https://doi.org/10.1007/s00253-017-8676-8

APPLIED GENETICS AND MOLECULAR BIOTECHNOLOGY

A novel DMAPP-responding genetic circuit sensor for high-throughput screening and evolving isoprene synthase

Chun-Li Liu[1] · Jing-Yi Cai[1] · Hao-Ran Bi[1] · Tian-Wei Tan[1]

Received: 6 July 2017 / Revised: 19 November 2017 / Accepted: 24 November 2017

Abstract

High-throughput screening is a popular tool for collating biological data which would otherwise require the use of excessive resources. In this study, an artificial genetic circuit sensor responding to dimethylallyl diphosphate (DMAPP) was constructed based on a modified L-arabinose operon for high-throughput screening and isoprene synthase (*ispS*) evolution in *Escherichia coli* (*E. coli*). As a first step, the DNA sequence of the L-arabinose ligand-binding domain (LBD) was replaced with an *ispS* gene to enable the AraC operon responding to DMAPP, which is the substrate of the IspS enzyme. Then, an enhanced GFP (eGFP) was also introduced as a reporter for pBAD promoter. The expression level of the reporter was monitored using either of the two tools: flow cytometer (FCM) and microplate reader. Sequentially, we observed that a high DMAPP concentration led to low eGFP fluorescence, and the overexpression of *ispS* gene, which consumes DMAPP, resulted in a high eGFP expression. These results demonstrated that the artificial genetic circuit sensor responded directly to the intracellular concentration of DMAPP, and the expression of IspS enzyme could be positively correlated to the expression level of eGFP. Finally, we identified two IspS mutants with different activities from an *ispS* gene library and further validated the screening method.

Keywords Genetic circuit sensor · DMAPP · L-arabinose · Fluorescence · Screening *ispS*

Introduction

Rubbers have got a very large global demand for the production of automobile tires, medical supplies surgical gloves, motor mounts and fittings, rubber bands, golf balls, and shoes. An important variety of rubber is a polymer of isoprene (2-methyl-1,3-butadiene). Almost all isoprene is petroleum-derived and thus subject to volatility in pricing and supply. Therefore, a reliable biological process for isoprene production utilizing renewable feedstock would be an industry-redefining development. Some bio-based processes for producing isoprene have been well studied in model organisms such as *Escherichia coli* (Liu et al. 2014) and yeast (Hong et al. 2012). Isoprene is biosynthesized from two basic precursors, isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP), in the presence of isoprene synthase (*ispS*) gene, which was originally identified from *Populus alba* or *kudzu* (Sasaki et al. 2005; Sharkey et al. 2001). There are two different pathways of isoprene production: the methyl erythritol 4-phosphate (MEP) pathway and the mevalonate (MVA) pathway (Adam and Zapp 1998; Mahmoud and Croteau 2001; Yoon et al. 2006) (see Fig. 1i). MVA pathway mainly exists in eukaryotes, archaebacteria, and cytosols of higher plants, while the MEP pathway exists in many eubacteria, cyanobacteria, and chloroplasts of higher plant (Hemmerlin et al. 2012). The heterologous expression of MVA pathway in *E. coli* has been reported to improve the production of isoprene and other terpenoids (Liu et al. 2015; Yang et al. 2012; Ozaydin et al. 2013).

Various efforts of pathway optimization have been made to obtain high isoprene production. As reported, the highest titer of isoprene was 60 g/L after fed-batch cultivation of engineered *E. coli* (Whited et al. 2010) in prokaryotes and 23.6 g/L after the aerobic fed-batch fermentation of

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00253-017-8676-8) contains supplementary material, which is available to authorized users.

✉ Tian-Wei Tan
twtan@mail.buct.edu.cn

[1] National Energy R&D Center for Biorefinery, Beijing Key Laboratory of Bioprocess, College of Life Science and Technology, Beijing University of Chemical Technology, Chaoyang District North Sanhuan Road no. 15, Beijing 100029, People's Republic of China

Published online: 14 December 2017

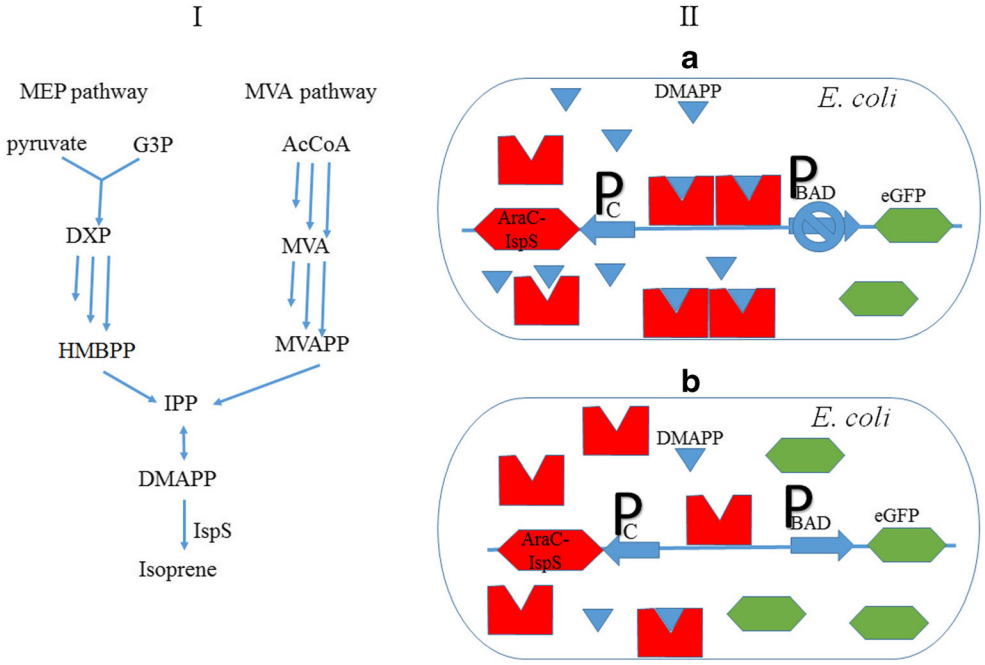


Fig. 1 **I** The two pathways for isoprene production: MEP pathway and MVA pathway. G3P, glyceraldehyde-3-phosphate; DXP, 1-deoxy-D-xylulose 5-phosphate; HMBPP, (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate; AcCoA, acetyl coenzyme A; MVA, mevalonate; MVAPP, mevalonate-5-diphosphate; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl pyrophosphate. **II** The synthetic genetic circuit sensor responding to DMAPP. Blue triangles stand for DMAPP; red shapes represent IspS-AraC gene or fusion protein; green shapes mean eGFP protein or gene. A = When the concentration of DMAPP is high, the fusion protein IspS-AraC dimerizes when binding to DMAPP, inactivating pBAD transcription. B = When the concentration of DMAPP is low, the fusion protein IspS-AraC becomes a monomer when dissociating with DMAPP, activating pBAD transcription

engineered yeast (Wang et al. 2017) in eukaryotes. Neither has reached a profitable level for industrial application. Enzyme IspS is one of the key components for isoprene production (Whited et al. 2010); however, the specific activity of IspS was generally low, such as 0.075, 0.5, and 0.16 U/mg in plant kudzu (recombinant) (Berry et al. 2002), aspen (native) (Julsing et al. 2007) and poplar (recombinant) (Floras et al. 1996), respectively. Because of the poor kinetics of these enzymes, protein engineering is required to achieve an isoprene production required of a commercially viable process. Thus, the highest possible specific activity of the IspS enzyme is crucial for overproduction of isoprene. In this report, a simple and fast method of screening and quantifying the activity of the isoprene biosynthesis enzymes is proposed.

An artificial genetic circuit capable of sensing target substrate is frequently applied to the development of screening methods to get the desired variants of the target enzyme. Chou et al. had established a feedback-regulated phenotypic evolution system to evolve IPP isomerase (Idi) for overproduction of desired metabolite (Chou and Keasling 2013). In nature, many examples of genetic circuit sensors consist of an inducer, a regulator, and a reporter, of which the inducer makes the conformation of the regulator change, and then induces the reporter expression, such as the lactose, L-arabinose, and galactose operons. AraC is a transcription regulator that undergoes conformational changes and dissociates from its special DNA-binding partner when it forms a complex with its monosaccharide ligand, L-arabinose. In absence of L-arabinose, a single AraC dimer binds with two widely separated half-sites (I_1 site at −35 and O_2 site at −285), forming a 210–base pair DNA loop and repressing the transcription from the pBAD promoter (Dunn and Schleif 1984; Hendrickson and Schleif 1985; Lobell and Schleif 1990; Martin et al. 1986). When L-arabinose is present, its binding to AraC causes the protein to cease DNA looping and favors binding to the adjacent two close half-sites I_1 and I_2 (close to −35 site), thus the transcription is activated (Hendrickson and Schleif 1984; Martin et al. 1986). The 292-residue AraC protein consists of a NH_2-terminal L-arabinose-binding domain (LBD, residues 1 to 170) that binds to L-arabinose and mediates AraC dimerization, a COOH-terminal DNA-binding domain (DBD, residues 178 to 292) and a linker of at least five amino acid residues which connects LBD and DBD (Bustos and Schleif 1993; Eustance et al. 1994; Eustance and Schleif 1997; Lauble et al. 1989). If the L-arabinose-binding domain is replaced with an enzyme of interest, the binding of the enzyme to its substrate may trigger a conformational change. If one of the binding conformations can activate the pBAD promoter, the transcription strength would be correlated to the concentration of the substrate of the enzyme.

The crystal structure of IspS observed upon binding its substrate DMAPP exhibits structural changes at the IspS

dimer interface. This dimer was formed by two additional hydrogen bonds and four additional salt bridges between the monomers, suggesting the existence of at least two different conformations (Koksal et al. 2010). Based on this observation, it would be possible to fuse IspS with the COOH-terminal domain of AraC to obtain a DMAPP sensor. In light of the AraC mechanism, we can modify the AraC operon by replacing the LBD sequence of this regulator with *ispS* gene, and construct a genetic circuit sensor composed of two parts: the new fused protein IspS-AraC and a pBAD-driven enhanced GFP (eGFP) as the reporter (see Fig. 1). It is then possible to use it for screening high activity IspS enzyme mutants that catalyzes the conversion of DMAPP to isoprene.

In this study, a DMAPP-responding genetic circuit sensor was first developed by replacing the LBD sequence of the regulator AraC with the *ispS* gene so that the expression of the reporter gene *eGFP* under control of the pBAD promoter can respond to DMAPP, instead of L-arabinose. The sensor's performance was then tested by monitoring the expression level of eGFP in *E. coli* BL21 (DE3) with DMAPP in vivo. The relationship between DMAPP concentration and eGFP fluorescence was examined by changing the concentrations. The regulation of this sensor by DMAPP was totally different from the L-arabinose induction mechanism. Thus, DMAPP acted as a repressor instead of inducer. Most of the intracellularly expressed fusion protein IspS-AraC bound to DMAPP when concentration of DMAPP was high. The interaction of IspS-AraC and DMAPP will lead to a conformational change of the fusion protein and repress the transcription from pBAD promoter. When *ispS* gene was overexpressed, consumption of its substrate DMAPP caused the dissociation of IspS-AraC homodimer and the activation of the downstream reporter of pBAD promoter. The changes of fluorescent level of eGFP induced by the dimerization of IspS-AraC were monitored by microplate reader and flow cytometer (FCM). We also demonstrated that this novel genetic circuit could serve as a simple and fast tool to screen and evolve *ispS* gene through screening two enzymes of different activities.

Materials and methods

Material and strains

Restriction enzymes, ligase, and DNA polymerase including Primer STAR HS and Ex TaqDNA were purchased from New England Bio-labs (Ipswich, MA) or TAKARA (Dalian, China). DMAPP standard (Sigma-Aldrich) was used for testing the correlation of its concentration with the output of eGFP florescence. Most of the chemicals, solvents, and media components were purchased and used without modification from Sigma-Aldrich (St. Louis, MO), Fisher Scientific (Pittsburgh, PA), or NEB (Ipswich, MA) unless otherwise noted. *E. coli* strains Top10 and BL21 (DE3) were used for plasmid construction and production experiments, respectively. All plasmids and strains listed in Table 1 were sequenced to verify the cloning accuracy.

Plasmids construction and screening of mutant IspS library

The plasmids for genetic circuit sensor were constructed based on the modified vector backbone pRSFDuet-1 with RSF Ori and kanamycin resistance gene. Two T7 expression cassettes were replaced by AraC operon with the eGFP inserted behind pBAD promoter. The vector backbone fragment was modified by digestion using *Xba* I and *Bgl* II enzymes. The DNA fragment pBAD-eGFP was generated by overlap PCR using the primers PBAD-F and eGFP-R. The fragment of DBD including the linker was connected with *ispS* gene through overlap PCR to obtain IspS-AraC fragment. The PCR programs were performed in a Mastercycler Personal (Eppendorf) with Primer STAR HS DNA Polymerase. Finally, the plasmid RSF-IspSC-eGFP (Fig. S2) was constructed via assembling the three DNA fragments of the vector bone fragment, pBAD-eGFP and IspS-AraC using a commercial Gibson Assembly® Master Mix kit (New England Biolabs). Plasmid RSF-IspSTerC-eGFP was constructed based on plasmid RSF-IspSC-eGFP by inserting a rrnB terminator between *ispS* and *AraC* through PCR with primers rrBTer-IspSAraC-F and rrBTer-IspSAraC-R and cyclization of the PCR product by T4 polynucleotide kinase and T4 ligase after digestion with enzyme Dpn I. Plasmid pTrc99a with ColE1 (pBR322) ori and ampicillin resistance gene was used for expressing *ispS* gene driven by pTrc promoter. The *ispS* gene was cloned with the primers IspS-*Nco*I-F and IspS-*Sac*I-R using the template of *E. coli* codon-optimized *ispS* gene (GenBank No. AB198180) from *P. alba* (Liu et al. 2014, 2015). The *ispS* gene without its predicted signal peptide was inserted into the pTrc99a vector between *Nco* I and *Sac* I sites (Fig. S2).

Randomly mutated *ispS* sequences were generated by error-prone PCR using ExTaq Mix DNA polymerase (TAKARA) with the concentration of Mn^{2+} was 0.3 mM in the PCR reaction. Then they were inserted into pTrc99a vector between *Nco* I and *Sac* I sites for constructing the IspS mutant library. The library was transformed into the screening host strain AraC-IS, and three plates with colonies were used for screening. Number of colonies on each plate was estimated to around 10^2–10^3. About 450 colonies were inoculated into 2 mL LB medium with 50 mg/L kanamycin, 100 mg/L ampicillin antibiotics, and 0.1 mM isopropyl b-D-1-thiogalactopyranoside (IPTG) in 48-well deep plates and then they were cultured for 48 h. The eGFP fluorescence of all the cultures was screened in 96-well plates using the microplate reader at time points 14, 23, and 38 h. The colonies identified with great deviations of eGFP florescence per OD_{600} comparing to strain IS-IC were selected for colony PCR and sequence.

Table 1 Plasmids and strains used in this study

Name	Relevant genotype	Reference
pRSFDUET-1	RSF Ori Kanar	This study
pTrc99a	pBR322 ori, AmpR,	This study
pTrc99a-IspS	pBR322 ori, AmpR,	This study
RSF-IspSC-eGFP	RSF Ori Kanar, IspS-AraC, PBAD-eGFP	This study
RSF-IspSTerC-eGFP	RSF Ori Kanar, IspS-rrnB Ter-AraC, PBAD-eGFP	This study
Strains	Description	Reference
E. coli TOP10	Applied for harvesting plasmid	Purchased from Biomed
E. coli BL21 (DE3)	Applied for expression genes	Purchased from Biomed
AraC-IS	RSF-IspSC-eGFP	This study
IC	pTrc99a+ RSF-IspSC-eGFP	This study
IS-IC	pTrc99a-IspS+ RSF-IspSC-eGFP	This study
NC	*E. coli* BL21 (DE3) harboring pRSFDuet-1	This study
IspS	pTrc99a-IspS	This study
AraC-ISTer	RSF-IspSTerC-eGFP	This study
IS-IC-31	pTrc99a-IspS with 345–349 residue truncated + RSF-IspSC-eGFP	This study
IS-IC-34	pTrc99a-IspS V93D R116H L148Q A172T F180S L272R M337 T L421Q I478F+ RSF-IspSC-eGFP	This study

Characterization of the fluorescence of eGFP with FACS.

Three colonies harboring the plasmid RSF-IspSC-eGFP were inoculated into LB medium with 50 mg/L kanamycin and cultured overnight as seed cultures. Then, each of them was diluted into two 4-mL fresh LB medium with the antibiotics to an initial optical density (OD_{600} nm) of 0.05 the following day. After culturing for 3 h, DMAPP was supplemented into one of the two tubes to a final concentration of 10 μM as the experimental group and equal volume of water was added into the other tubes as the control group. After culturing for another 21 h at 30 °C, the cultures were diluted to OD_{600} of 0.1 or a cell density of 10^6/mL using deionized water and then they were filtered into the 15-mL tubes by etamine. Finally, samples were shot into FACSAria II (BD, Franklin Lakes, NJ) for eGFP fluorescence detection. The FACS was performed with the excitation at 488 nm and the emission at 509 nm for detecting eGFP fluorescence. Fluorescein (FITC) channel was used for sorting target cells in the total number of cells and eGFP gating for calculation the positive numbers. The FACS flow sheath fluid (BD, Franklin Lakes, NJ) was applied for keeping the sample flow constant.

Measurement of eGFP fluorescence through microplate reader.

Colonies harboring plasmid RSF-IspSC-eGFP and empty pTrc99a, and colonies harboring plasmid RSF-IspSC-eGFP and pTrc99a-IspS on plates were inoculated into LB medium with 50 mg/L kanamycin and 100 mg/L ampicillin antibiotics. Starter cultures were grown overnight at 37 °C, and then every culture was diluted into three or four tubes with fresh LB culture containing antibiotics, respectively. After culturing for 3 h at 37 °C, they were supplemented with 0.1 mM IPTG or 10 mg/L L-arabinose when OD_{600} reached 0.6, and allowed to continue growth for 35 h at 30 °C. At time points 14, 23, and 38 h, 100-μL culture was sampled and diluted 10 times with LB medium for eGFP florescence and OD_{600} detection in a 96-well plate. The eGFP fluorescence was measured by microplate reader Spectramax M2 set of excitation at 488 nm and emission at 509 nm. The eGFP florescence per OD_{600} after removing the background was compared for analyzing of the responding of the genetic circuit sensor to *ispS* expression.

Isoprene production and detection.

Three colonies were inoculated into 4 mL LB medium, cultured at 37 °C as the seed cultures and then diluted into the fresh medium in 20-mL sealed vials to produce isoprene. The *E. coli* cell cultures were incubated in rotary shakers (200 rpm) at 30 °C after 0.5 mM IPTG was added at OD_{600} of 0.6–0.8. After culturing for 48 h, 1 mL of gas sample from the 20-mL sealed vials was injected into a gas chromatography mass spectrometry (GC-MS) by the headspace auto-injector for detecting isoprene production. The GC-MS (Thermo Fisher, Trace ISQ America) equipped with an electron impact (EI) detector and a TG-WAXMS column (30 m × 0.25 mm × 0.25 um film thickness) was configured to the following conditions: inlet at 200 °C, 1.1 mL/min constant flow, transfer line at 300 °C, ion source at 250 °C, and scan *m*/*z* 50–300. The

oven temperature programs were set to the initial temperature of 40 °C for 1 min, then increasing to 200 °C at 15 °C/min rate, and finally holding for 1 min. The injector was maintained at 220 °C. The samples were incubated at 60 °C, subjected to a 10-min cycle of 10-s shaking and 10-s keeping still before injection with the headspace needle at 70 °C. The sample drawn was 1 mL and split in the column with a ratio of 1:50.

Results

Construction and the structure analysis of genetic circuit sensor.

This artificial genetic circuit sensor was constructed based on the L-arabinose operon, in which the LBD was replaced by *ispS* gene. The *eGFP* gene was inserted under the control of pBAD promoter (the design was shown in Fig. S1). If the protein IspS undergoes a conformational change when bound to its substrate DMAPP, the hypothesis is that only one of the two possible conformations may activate the transcription of pBAD promoter and induce eGFP expression.

To our knowledge, AraC regulates expression of L-arabinose utilization genes from the L-arabinose-inducible promoter pBAD by preferentially binding to different DNA sequences in the presence and absence of L-arabinose. AraC has a distinct amino-terminal LBD and carboxy-terminal DNA-binding domain DBD, and the activation and repression of pBAD depend on whether the LBD has bound with L-arabinose (Koksal et al. 2010). To this end, the plasmid RSF-IspSC-eGFP was constructed by inserting the *ispS* gene from *P. alba* between the pC promoter and the linker region of AraC (170–178 amino acid) based on the modified vector pRSFDuet-1. To further illustrate that eGFP expression was regulated by fusion protein IspS-AraC, an rrnB terminator was inserted between *ispS* and AraC sequence to construct plasmid RSF-IspSTerC-eGFP. In addition, to study the relationship between *ispS* expression and eGFP florescence, plasmid pTrc99a-IspS was constructed for expressing *ispS* gene which led to change of the concentration of DMAPP.

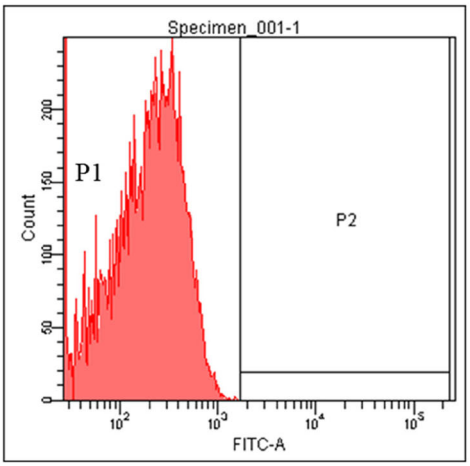
eGFP has been validated as a reporter by FACS.

The fused protein IspS-AraC should be in dimeric conformation when binding to DMAPP and in monomeric conformation when separating with DMAPP. However, we did not know which conformation could induce the reporter gene expression. If the binding of the fusion protein IspS-AraC with DMAPP can induce the expression of proteins under the control of pBAD promoter, the fluorescence of eGFP will increase with higher concentration of DMAPP. Then DMAPP will act as an inducer like L-arabinose in the original AraC-operon. Otherwise, the binding may repress the expression of eGFP protein, then the fluorescence will decrease with higher concentration of DMAPP.

To study the relationship between the expression level of eGFP and the concentration of DMAPP, we first cultured the seed cultures of negative control, AraC-IS and AraC-ISTer strain, and then diluted every one into two tubes with fresh LB culture. The two tubes of every strain were, respectively, supplemented with 10 μM DMAPP and equal volume of water when OD_{600} reached 0.6. Sequentially, we used FACS to monitor the fluorescence of eGFP from the two cultures respectively with additional DMAPP and water after the cultures were diluted with deionized water. In FACS histograms, FITC-A mean is the indicator of eGFP fluorescent level, and the number of cells in P2 area stands for the positively staining population with high fluorescence. The percentages of the cells in P2 area represent the ratio of cells with higher fluorescence intensity of eGFP.

According to the analysis, we obtained the percentages of the positively staining population [P2/(P1 + P2) (% parent)], the mean fluorescence intensity of the positively staining population [P2 (FITC-A mean)] and of all the cell populations [P1 + P2 (FITC-A mean)]. As shown in Fig. 2a, e, c, the percentages of cell populations in P2 area were, respectively, 0, 1.1, and 34.9% in the cultures of negative control, AraC-ISTer and IspS-AraC without additional DMAPP. The percentages of the three strains indicated that the eGFP expression under pBAD promoter considerably decreased without fusion protein IspS-AraC. The eGFP fluorescence of negative control showed no difference between the cultures with and without DMAPP (Fig. 2a, b), indicating that DMAPP had no effect on the eGFP fluorescence without fusion protein IspS-AraC. The percentage of the positively staining population [P2/(P1 + P2) (% parent)] of the sample with additional DMAPP was 21.7% (Fig. 2d), decreased 38% when compared with the strain IspS-AraC without DMAPP (Fig. 2c). Moreover, in the culture without additional DMAPP, the highest FITC-A mean reached to 10^5, and two peaks were observed, of which the second peak in P2 area represented the cells with high eGFP fluorescence. While with the addition of 10 μM DMAPP, the highest FITC-A mean was about 10^4, and the peak in P2 area disappeared, indicating that the eGFP fluorescence decreased sharply. These results indicated that the eGFP expression was lower with higher DMAPP concentration; therefore, DMAPP acted as a suppressor for promoter pBAD. In comparison of the eGFP fluorescence in Fig. 2c, d, the fluorescence of strain IspS-AraC supplemented with 10 μM DMAPP was 38% lower than that supplemented with equal volume water, supporting that high-concentration DMAPP can repress pBAD promoter transcription. Therefore, the dimer conformation binding with DMAPP can repress pBAD promoter and this eGFP-based artificial genetic circuit sensor was initially verified to be a sensitive DMAPP-responding system using

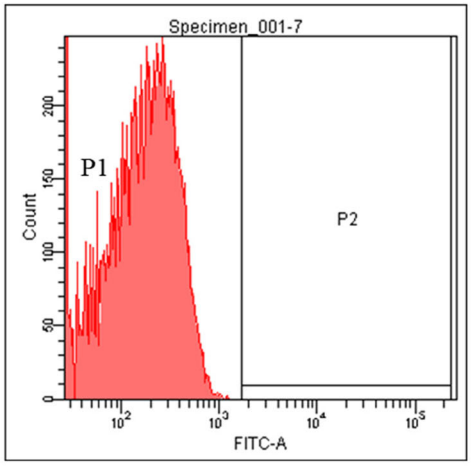
a



NC -DMAPP

Population	#Events	%Parent	FITC-A Mean
P1	19,957	40.1	80
P2	0	0.0	####

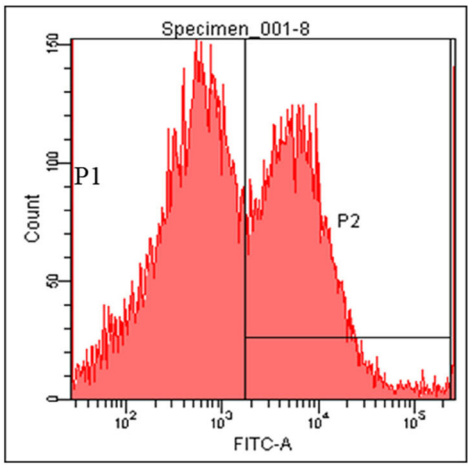
b



NC +DMAPP

Population	#Events	%Parent	FITC-A Mean
P1	20,000	25.0	83
P2	0	0.0	####

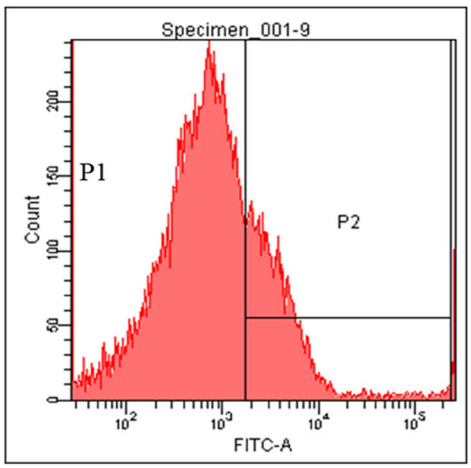
c



AraC-IS -DMAPP

Population	#Events	%Parent	FITC-A Mean
P1	19,155	10.9	8,356
P2	6,680	34.9	11,545

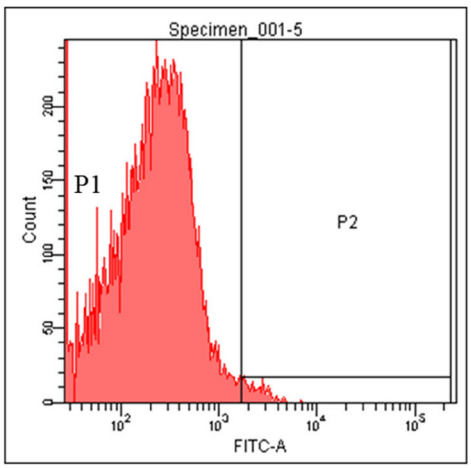
d



AraC-IS +DMAPP

Population	#Events	%Parent	FITC-A Mean
P1	19,465	8.7	5,405
P2	4,228	21.7	8,807

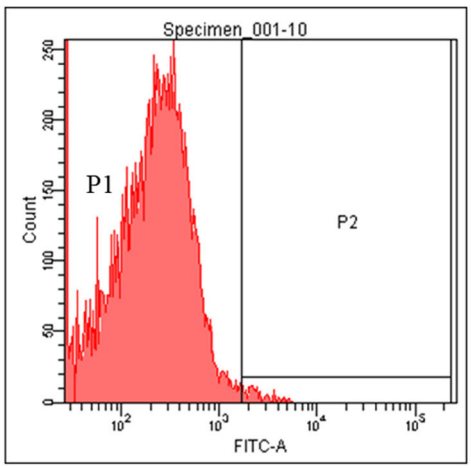
e



AraC-ISTer -DMAPP

Population	#Events	%Parent	FITC-A Mean
P1	20,000	32.7	164
P2	215	1.1	2,541

f



AraC-ISTer +DMAPP

Population	#Events	%Parent	FITC-A Mean
P1	20,000	34.5	148
P2	166	0.8	2,697

◀ **Fig. 2** FACS histogram of negative control, AraC-IS, and AraC-ISTer for eGFP fluorescence analysis. **a**, **c**, and **e** The cultures of strain negative control (NC), AraC-IS, and AraC-ISTer were supplemented with water. **b**, **d**, and **f** The cultures of strain NC, AraC-IS, and AraC-ISTer were supplemented with 10 μM DMAPP. All the strains were shot for FACS after cultured 24 h at the same condition. The FACS was performed with the excitation at 488 nm and the emission at 509 nm for eGFP fluorescence. The number of cells in the P2 area stands for the positively staining population with high fluorescence

FACS technology. The FACS histogram of strain AraC-ISTer (Fig. 2e, f) demonstrated that almost no eGFP fluorescence was detected after the fusion protein IspS-AraC was broken by the rrnB terminator, indicating that the regulation of eGFP expression degenerated without the fusion protein. The fusion protein IspS-AraC has also been verified to be capable to catalyze DMAPP into isoprene but its activity decreased since isoprene production of strain AraC-IS was lower than strain AraC-ISTer (shown in supplementary Fig. S4).

Analysis of eGFP fluorescence responding to ispS expression.

After confirming that the eGFP fluorescence was related to DMAPP concentrations through FACS, we would like to investigate that the intensity of eGFP fluorescence could be linked to *ispS* gene expression, using a microplate reader to monitor the fluorescence. In order to prove the eGFP fluorescence of the cells was distinguishably affected by the expression of *ispS* gene, we respectively transformed plasmid pTrc99a and pTrc99a-IspS into *E. coli* IspS-AraC, obtaining IC as the control strain and IS-IC as the experiment strain. Plasmid pTrc99a-IspS was constructed for *ispS* gene overexpression with IPTG-induced pTrc promoter, since its overexpression would cause the concentration of DMAPP to change. To support that the overexpression of *ispS* gene could induce the eGFP fluorescence changing, we detected the output of eGFP reporter of the two strains, respectively, supplemented with IPTG, L-arabinose, or both, of which L-arabinose was used for proving the inducer changing into DMAPP.

In Fig. 3, no significant difference has been observed on the eGFP fluorescence across three sampling time points of 14, 23, and 38 h not only in the IPTG presence culture and IPTG absence culture, but also in the presence of both 0.1 mM IPTG and 10 mg/L L-arabinose culture and the culture with only 10 mg/L L-arabinose in control strain IC. These results indicated that IPTG itself had no influence on the genetic circuit sensor system without *ispS* gene overexpression. For strain IS-IC, the eGFP fluorescence was five times higher with 0.1 mM IPTG and seven times higher with 0.1 mM IPTG and 10 mg/L L-arabinose. These results indicated that the overexpression of *ispS* gene instead of L-arabinose became the main factor of regulating the reporter eGFP expressing in strain IS-IC. After adding 0.1 mM IPTG, the overexpressed IspS enzyme consumed the substrate DMAPP in vivo, and then induced the expression of eGFP. It was well aligned with the previous conclusion that low concentration of DMAPP induced the eGFP expression.

In conclusion, the fusion protein IspS-AraC did not have enough DMAPP to bind with in the low level of DMAPP resulting from overexpression of *ispS* gene, and then promoter pBAD was activated and the fluorescence of eGFP was higher than that without *ispS*. The concentration of DMAPP became the major factor that can change the expression strength of gene eGFP under pBAD promoter in this genetic circuit sensor. Moreover, the expression level of IspS had a decisive effect on the expression strength of eGFP by consuming DMAPP. Thus, the higher activities of IspS leads to the lower concentration of DMAPP and higher eGFP fluorescence. Based on the above analysis, the DMAPP-responding genetic circuit sensor was established and verified successfully.

Screen different activity enzyme IspS.

To apply this method to screening IspS (Fig. 4), we generated the IspS mutant library by error-prone PCR before subjecting to eGFP fluorescence measurement. We also tested the relative production of isoprene produced by strain IC, IC-IS, and the screened mutants. As a result, although most of the mutants showed constantly similar fluorescence with the wild-type strain, several colonies showed significant negative fluorescence offset. Two of the negative samples with the lowest fluorescence IS-IC-31 and IS-IC-34 were sequenced, revealing the mutation sites 345–349 residue truncated, and nine amino acids were mutated: V93D, R116H, L148Q, A172T, F180S, L272R, M337T, L421Q, and I478F.

As shown in Fig. 5, compared with the wild-type strain IS-IC, the fluorescence and the isoprene production of strain IS-IC-31 were decreased simultaneously by 52.7 and 93%, respectively, and for strain IS-IC-34, the fluorescence was decreased to the level of negative control IC. These results indicated that the activities of IspS enzymes with 345–349 residue truncated or the nine amino acids mutated were considerably lower than the wild recombinant IspS enzyme, and eGFP fluorescence is a useful reporter of *ispS* gene activity. According to Fig. 5a, b, we can see that strain IS-IC-34 and IC performed similar eGFP fluorescence and isoprene production, indicating that the IspS enzyme activity in IS-IC-34 was probably inactive. After analysis of the mutations, we found the amino acids of 345–349 were reported to be responsible for binding of Mg^{2+} which is a cofactor in IspS enzyme catalysis (Sasaki et al. 2005), and the truncations of these amino acids in strain IS-IC-31 were suspected to reduce the enzyme activity by weakening the binding of Mg^{2+}. The nine amino acids in strain IS-IC-34 changed from aliphatic amino acid V, R, L, A, I, and L to hydrophilic amino acids, probably resulting to the inactivation of IspS enzyme. In summary, this work provided a promising tool for IspS screening and further validated this screening method.

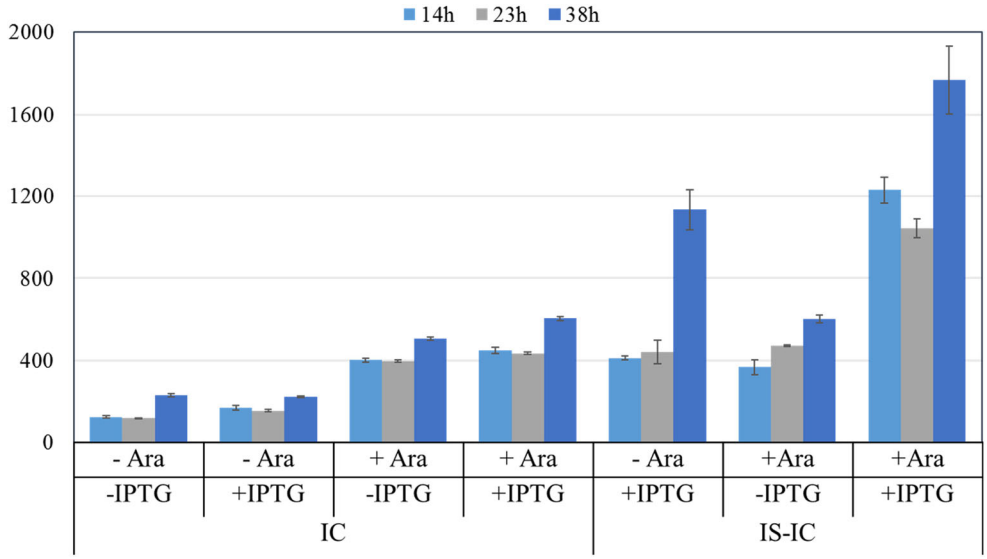


Fig. 3 PBAD output from the genetic circuit sensor responding to DMAPP in strain IC and IS-IC. IC is the control without the additional *ispS* expression, and IS-IC harboring RSF-IspSC-eGFP and pTrc99a-IspS plasmid is for studying the relationship of eGFP fluorescence and *ispS* gene expression. The eGFP florescence of the cultures was monitored by microplate reader after they were induced by 0.1 mM IPTG at time points of 14, 23 and 38 h. + or − Ara means with/without L-arabinose supplemented in the cultures. IPTG and L-arabinose were supplemented when OD_{600} reaches to 0.6. EGFP fluorescence was measured at the excitation of 488 nm and the emission of 509 nm using microplate reader Spectramax M2. Data represent averages from three replicate cultures and error bars show s.d.

Discussion

In this study, we successfully developed a genetic circuit biosensor based on the L-arabinose operon to screening for a highly active IspS enzyme. In brief, we can screen cells through two methods: one is sorting the cells with high eGFP florescence through FACS, the other is using a microplate reader to monitor the eGFP fluorescence of the cultures

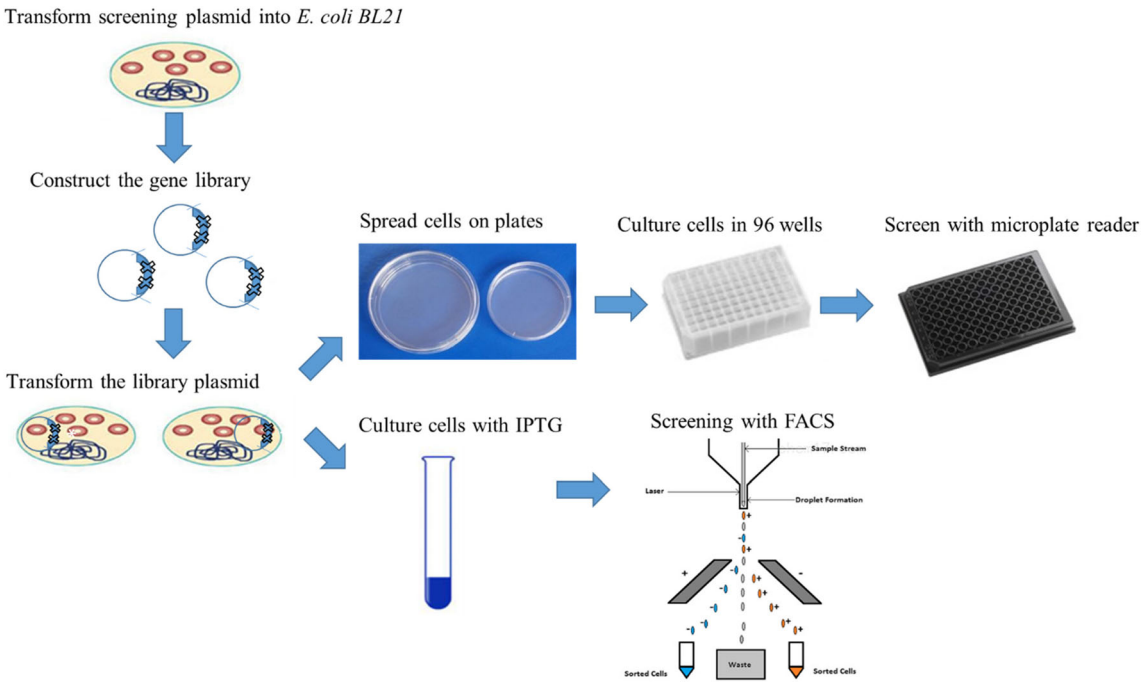


Fig. 4 The flow path and two methods of screening IspS enzyme. Randomly mutated *ispS* sequences were generated by error-prone PCR using ExTaq Mix polymerase with 0.3 mM Mn^{2+}, and then ligated into plasmid pTrc99a to construct the *ispS* gene library. The library plasmid pTrc99-IspS was co-transformed with screening plasmid RSF-IspSC-eGFP into *E. coli* BL21 (DE3) for screening mutants. The desired mutants were screened by measuring the eGFP fluorescence through two methods: one is using microplate reader to screen the IPTG-induced cultures in 96-well plate, the other is sorting the IspS mutants with FACS after cultivation and induction with IPTG

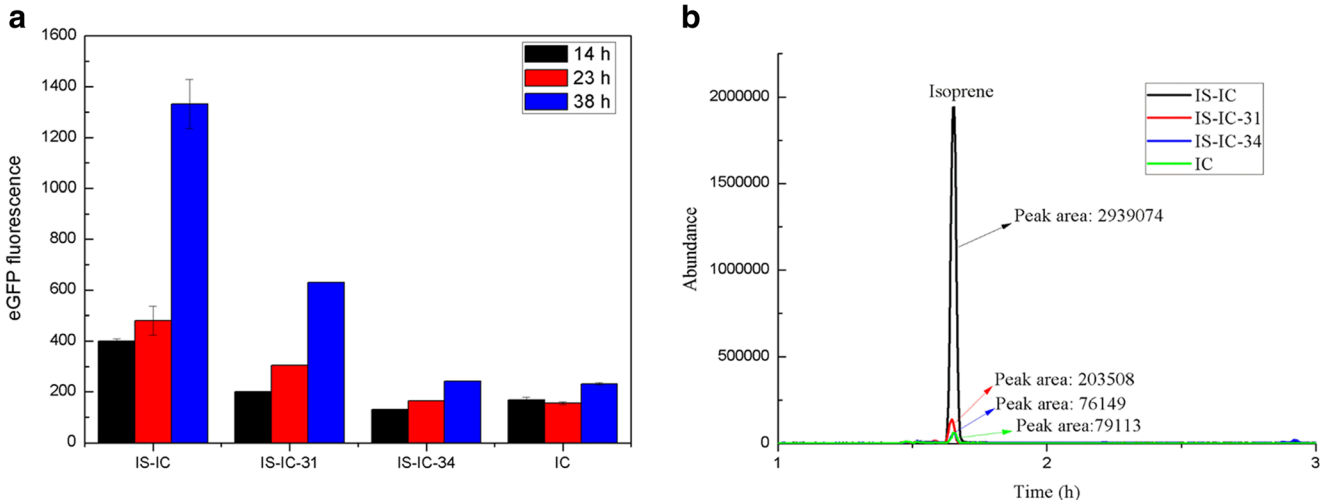


Fig. 5 The characterization of the screened IspS enzyme with different activities. **a** The eGFP fluorescence of strain IS-IC, IC, IS-IC-31, and IS-IC-34. The florescence was measured by microplate reader at time point 14 h (black), 23 h (red), and 38 h (blue). **b** The relative production of isoprene in strain IS-IC (black), IC (green), IS-IC-31 (red), and IS-IC-34 (blue). The isoprene production was detected by GC-MS with headspace sample injector after the cultures were induced with 0.1 mM IPTG in rotary shakers (200 rpm) at 30 °C for 48 h. The peak area reveals the isoprene production levels

after induction by 0.1 mM IPTG in the 96-well deep plate format (see Fig. 4). It is easy to operate since large amounts of cells could be sorted with FCM in a relative short period of time and 96 colonies can be screened in one plate with the microplate reader. Both of these methods employing the genetic circuit sensor are more feasible, sensitive, and simple for isolating the desired mutants from a gene library than the method involving of pyrolysis-gas chromatography/mass spectrometry to screen and analysis isoprene and limonene released from polyprenols and polyisoprenes (Takeno et al. 2010), or some other methods based on the color change of the cells harboring carotenoid pathways (Furubayashi et al. 2014).

In summary, we demonstrated the application of a genetic circuit sensor designed by modifying L-arabinose operon, confirmed the sensor's responding to DMAPP, and identified two mutants by monitoring the actuator level with eGFP protein. Based on the analysis of FACS and microplate reader, we found that the regulation mechanism of IspS-AraC fusion was completely different from that of the L-arabinose operon. In the later regulation, L-arabinose is an inducer, to activate promoter pBAD transcription and gene expression. However, in this genetic circuit sensor, DMAPP acted as a repressor, to repress promoter pBAD transcription and the gene expression when the concentration of DMAPP was high. To explain the negative correlation between eGFP fluorescence and DMAPP concentration, it is supposed that when the concentration of the intracellular DMAPP is low, the fusion protein IspS-AraC in DMAPP-dissociated state is keeping monomer conformation which ceases DNA looping and hinders the position of the RNA polymerase, resulting in activation of promoter pBAD transcription. On the contrary, when the concentration of the intracellular DMAPP is high, the dimers are formed by two additional hydrogen bonds and four additional salt bridges between monomers according to the study of IspS protein (Koksal et al. 2010). In this case, we suspect that the dimer constructs, consisting of a pair of fusion proteins and a pair of fixed DMAPP molecular binding with DNA sites, are too large to occupy the space and obstruct the RNA polymerase binding to the DNA sites. In addition, DMAPP was found to be toxic to the cells when comparing the OD_{600} of the cultures with and without additional DMAPP (Fig. S3). This is consistent with a previously reported study (Martin et al. 2003; Sivy et al. 2011).

Although the use of the L-arabinose operon has been demonstrated before (Chou and Keasling 2013), reports of modification of this operon for application in high-throughput screening of high activity enzymes are rare. The application of this genetic circuit sensor might be extended also to quantitative screening of other isoprenoid biosynthesis related the enzymes of the MEP/MVA pathway through monitoring the DMAPP concentration. In addition, the modification of the lactose operon to construct a similar sensor which should in theory be viable in respond to DMAPP has been attempted. However, efforts to obtain a specific sensor after the attempts of replacing the specific lactose-binding domain in repressor LacI with IspS enzyme based on the structure analysis of LacI (Lewis et al. 1996) have failed. Further research would also be needed to evaluate the possible association of the substrate DMAPP and its binding domain in this biosensor using some other technologies, such as the immunofluorescence and electron microscope technologies. Establishing an enzyme screening system for high isoprene production might need considerable investment in work and time at a commercially relevant

cost, involving: (1) the construction of an *ispS* gene library, (2) screening and selecting high fluorescence cells or colonies, and (3) identifying the enzyme activity.

Isoprene is an important raw material in the synthetic chemistry industry and its biosynthesis has become one of the research hotspots in recent years. Furthermore, biocatalysts are recognized as one of the key components for "sustainable chemistry development," that requires new enzymes with higher chemical reactivity, specificity and stability. In the study of bioprocess for isoprene-monomer production by Genencor, it has been demonstrated that optimizing, engineering, and designing enzyme activities to mitigate the inhibition of the key intermediate or end product is one important step in making the isoprene commercial production (Whited et al. 2010). However, the *ispS* genes from plants were characterized by low activities (Berry et al. 2002; Julsing et al. 2007; Floras et al. 1996), thus exploring the IspS enzyme with high activity is of high priority for improving isoprene production. The screening method reported in this paper has got great significance in developing isoprene biosynthesis to industrialization.

Acknowledgements This work was supported by the State key laboratory of organic–inorganic composites, the National Basic Research Program of China (973 program) (2013CB733600), and the National Nature Science Foundation of China (21390202, 21476017). The authors also thank David Luo (Lawrence Berkeley National Lab, Joint BioEnergy Institute) and Jan-Christer Janson (Uppsala University) for revising the language of this manuscript.

Compliance with ethical standards

Conflict of interest The author(s) declare that they have no competing interests

Ethical statement This article does not contain any studies with human participants performed by any of the authors.

References

Adam KP, Zapp J (1998) Biosynthesis of the isoprene units of chamomile sesquiterpenes. Phytochemistry 48(6):953–959. https://doi.org/10.1016/S0031-9422(97)00992-8

Berry A, Dodge TC, Pepsin M, Weyler W (2002) Application of metabolic engineering to improve both the production and use of biotech indigo. J Ind Microbiol Biotech 28(3):127–133. https://doi.org/10.1038/sj.jim.7000228

Bustos SA, Schleif RF (1993) Functional domains of the AraC protein. Proc Natl Acad Sci USA 90(12):5638–5642

Chou HH, Keasling JD (2013) Programming adaptive control to evolve increased metabolite production. Nat Commun 4:2595. https://doi.org/10.1038/ncomms3595

Dunn TM, Schleif RF (1984) An operator at −280 base pairs that is required for repression of araBAD operon promoter: addition of DNA helical turns between the operator and promoter cyclically hinders repression. Proc Natl Acad Sci USA 81(16):5017–5020. https://doi.org/10.1073/pnas.81.16.5017

Eustance RJ, Schleif RF (1997) The linker region of AraC protein. J Bacteriol 178(24):7025–7030

Eustance RJ, Bustos SA, Schleif RF (1994) Reaching out. Locating and lengthening the interdomain linker in AraC protein. J Mol Biol 242(4):330–338

Floras N, Xiao J, Berry A, Bolivar F, Valle F (1996) Pathway engineering for the production of aromatic compounds in *Escherichia coli*. Nat Biotechnol 14(5):620–623. https://doi.org/10.1038/nbt0596-620

Furubayashi M, Ikezumi M, Jun K, Iwasaki M, Fujii A, Li L, Saito K, Umeno D (2014) A high-throughput colorimetric screening assay for terpene synthase activity based on substrate consumption. PLoS One 9(3):11

Hemmerlin A, Harwood JL, Bach TJ (2012) A raison d'etre for two distinct pathways in the early steps of plant isoprenoid biosynthesis? Prog Lipid Res 51(2):95–148. https://doi.org/10.1016/j.plipres.2011.12.001

Hendrickson W, Schleif RF (1984) Regulation of the *Escherichia coli* l-arabinose operon studied by gel electrophoresis DNA binding assay. J Mol Biol 178(3):611–628. https://doi.org/10.1016/0022-2836(84)90241-9

Hendrickson W, Schleif R (1985) A dimer of AraC protein contacts three adjacent major groove regions of the araI DNA site. Proc Natl Acad Sci USA 82(10):3129–3133. https://doi.org/10.1073/pnas.82.10.3129

Hong SY, Zurbriggen AS, Melis A (2012) Isoprene hydrocarbons production upon heterologous transformation of *Saccharomyces cerevisiae*. J Appl Microbiol 113(1):52–65. https://doi.org/10.1111/j.1365-2672.2012.05319.x

Julsing MK, Rijpkema M, Woerdenbag HJ, Quax WJ, Kayser O (2007) Functional analysis of genes involved in the biosynthesis of isoprene in *Bacillus subtilis*. Appl Microbiol Biotech 75(6):1377

Koksal M, Zimmer I, Schnitzler JP, Christianson DW (2010) Structure of isoprene synthase illuminates the chemical mechanism of teragram atmospheric carbon emission. J Mol Biol 402(2):363–373. https://doi.org/10.1016/j.jmb.2010.07.009

Lauble H, Georgalis Y, Heinemann U (1989) Studies on the domain structure of the *Salmonella typhimurium* AraC protein. Eur J Biochemist 185(2):319–325. https://doi.org/10.1111/j.1432-1033.1989.tb15118.x

Lewis M, Chang G, Horton NC, Kercher MA, Pace HC, Schumacher MA, Brennan RG, Lu P (1996) Crystal structure of the lactose operon repressor and its complexes with DNA and inducer. Science 271(5253):1247–1254. https://doi.org/10.1126/science.271.5253.1247

Liu C-L, Fan L-H, Liu L, Tan T-W (2014) Combinational biosynthesis of isoprene by engineering the MEP pathway in *Escherichia coli*. Proc Biochemist 49(12):2078–2085

Liu C-L, Lv Q, Tan T-W (2015) Joint antisense RNA strategies for regulating isoprene production in *Escherichia coli*. RSC Adv 5(91):74892–74898. https://doi.org/10.1039/C5RA12161B

Lobell RB, Schleif RF (1990) DNA looping and unlooping by AraC protein. Science (N.Y.) 250(4980):528–532

Mahmoud SS, Croteau RB (2001) Metabolic engineering of essential oil yield and composition in mint by altering expression of deoxyxylulose phosphate reductoisomerase and menthofuran synthase. Proc Natl Aacd Sci USA 98(15):8915–8920. https://doi.org/10.1073/pnas.141237298

Martin K, Huo L, Schleif RF (1986) The DNA loop model for ara repression: AraC protein occupies the proposed loop sites in vivo and repression-negative mutations lie in these same sites. Proc Natl Aacd Sci USA 83(11):3654–3658. https://doi.org/10.1073/pnas.83.11.3654

Martin VJJ, Pitera DJ, Withers ST, Newman JD, Keasling JD (2003) Engineering a mevalonate pathway in *Escherichia coli* for production of terpenoids. Nat Biotechnol 21(7):796–802. https://doi.org/10.1038/nbt833

Ozaydin B, Burd H, Lee TS, Keasling JD (2013) Carotenoid-based phenotypic screen of the yeast deletion collection reveals new genes with roles in isoprenoid production. Metab Eng 15:174–183. https://doi.org/10.1016/j.ymben.2012.07.010

Sasaki K, Ohara K, Yazaki K (2005) Gene expression and characterization of isoprene synthase from *Populus alba*. Febs Lett 579(11): 2514–2518. https://doi.org/10.1016/j.febslet.2005.03.066

Sharkey TD, Chen X, Yeh S (2001) Isoprene increases thermotolerance of fosmidomycin-fed leaves. Plant Physiol 125(4):2001

Sivy TL, Fall R, Rosenstiel TN (2011) Evidence of isoprenoid precursor toxicity in *Bacillus subtilis*. Biosci Biotechnol Biochem 75(12): 2376–2383. https://doi.org/10.1271/bbb.110572

Takeno S, Bamba T, Nakazawa Y, Fukusaki E, Okazawa A, Kobayashi A (2010) High-throughput and highly sensitive analysis method for polyisoprene in plants by pyrolysis-gas chromatography/mass spectrometry. Biosci Biotech Biochemist 74(1):13–17

Wang F, Lv X, Xie W, Zhou P, Zhu Y, Zhen Y, Yang C, Yang X, Ye L, Yu H (2017) Combining Gal4p-mediated expression enhancement and directed evolution of isoprene synthase to improve isoprene production in *Saccharomyces cerevisiae*. Metab Eng 39:257–266. https://doi.org/10.1016/j.ymben.2016.12.011

Whited MG, Feher JF, Benko AD, Cervin AM, Chotani KG, McAuliffe CJ, LaDuca JR, Ben-Shoshan AE, Sanford JK (2010) Development of a gas-phase bioprocess for isoprene-monomer production using metabolic pathway engineering. Industrial Biotech 6(3):152–163

Yang J, Xian M, Su S, Zhao G, Nie Q, Jiang X, Zheng Y, Liu W (2012) Enhancing production of bio-isoprene using hybrid MVA pathway and isoprene synthase in *E. coli*. PLoS One 7(4):e33509. https://doi.org/10.1371/journal.pone.0033509

Yoon SH, Lee YM, Kim JE, Lee SH, Lee JH, Kim JY, Jung KH, Shin YC, Keasling JD, Kim SW (2006) Enhanced lycopene production in *Escherichia coli* engineered to synthesize isopentenyl diphosphate and dimethylallyl diphosphate from mevalonate. Biotechnol Bioeng 94(6):1025–1032. https://doi.org/10.1002/bit.20912