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A Genetically Encoded Biosensor for Monitoring Isoprene Production in Engineered *Escherichia coli*

Seong Keun Kim,[†] Seo Hyun Kim,[†,∥] Bindu Subhadra,[†,⊥] Seung-Gyun Woo,[†,‡] Eugene Rha,[†] Seon-Won Kim,[§] Haseong Kim,[†,‡] Dae-Hee Lee,*[,†,‡] and Seung-Goo Lee*[,†,‡]

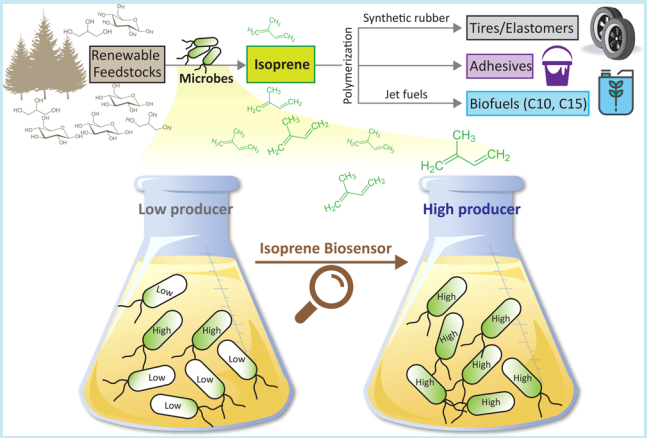
[†]Synthetic Biology and Bioengineering Research Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Daejeon 34141, Republic of Korea

[‡]Department of Biosystems and Bioengineering, KRIBB School of Biotechnology, University of Science and Technology (UST), Daejeon 34113, Republic of Korea

[§]Division of Applied Life Science (BK21 Plus), PMBBRC, Institute of Agriculture and Life Sciences, Gyeongsang National University, Jinju 52828, Republic of Korea

Supporting Information

ABSTRACT: Isoprene is a valuable precursor for synthetic rubber and a signature product of terpenoid pathways. Here, we developed an isoprene biosensor by employing a TbuT transcriptional regulator of *Ralstonia pickettii* to express a fluorescent reporter gene in response to intracellular isoprene in engineered *Escherichia coli*. The TbuT regulator recognizes isoprene as its less-preferred effector molecule; thus, we amplified the reporter gene expression using a T7 RNA polymerase-mediated transcriptional cascade and iteratively tuned the promoter transcribing *tbuT* to improve the sensitivity for detecting isoprene. When the engineered *E. coli* cells expressed heterologous genes for isoprene biosynthesis, the intracellular isoprene was expelled and the *tbuT* transcription factor was subsequently activated, leading to *gfp* expression. The chromosomal isoprene biosensor showed a linear correlation between GFP fluorescence and intracellular isoprene concentration. Using this chromosomal isoprene biosensor, we successfully identified the highest isoprene producer among four different *E. coli* strains producing different amounts of isoprene. The isoprene biosensor presented here can enable high-throughput screening of isoprene synthases and metabolic pathways for efficient and sustainable production of bioisoprene in engineered microbes.



KEYWORDS: *isoprene, biosensor, Escherichia coli, transcription factor, TbuT, T7 RNA polymerase-mediated transcriptional cascade*

Terpenoids, also known as isoprenoids, are relatively large and diverse chemicals that are produced naturally as plant secondary metabolites. They have been used as flavors and fragrances, and have the potential for use as biopharmaceuticals[1,2] and biofuels.[3] Terpenoids are derived in myriad ways from the assembly and modification of five-carbon isoprene units that are also key monomers for the production of synthetic rubbers and elastomeric materials.[4] In nature, plants secrete large quantities of isoprene; however, plants have not been used as an efficient host for isoprene production because the chemical is very volatile (boiling point 34 °C).[5] In this context, microbes can be an alternative for mass production of isoprene and have been used for biosynthesis of isoprene.[6,7] In particular, *Escherichia coli* has been used as a primary host in many studies to produce bioisoprene.[6−13] Identifying and optimizing the assembly of heterologous genes encoding the members of isoprene-production pathways[6,12,13] and finding the optimal conditions for isoprene production[7,11] are the main hurdles to overcome before improving isoprene production in *E. coli*. Thus, novel high-throughput screening systems for isoprene production should be developed to rapidly identify heterologous genes and their assembly to guarantee high-level bioisoprene production.

The lack of high-throughput screening tools often limits the engineering of enzymes or pathways for the biosynthesis of molecules of interest. This limitation can be overcome by biosensors that couple metabolite recognition with changes in reporter gene expression. Genetically encoded biosensors have been used to detect and quantify intracellular metabolites in a concentration-dependent manner.[14−17] In nature, various transcription factors have evolved to sense the concentration

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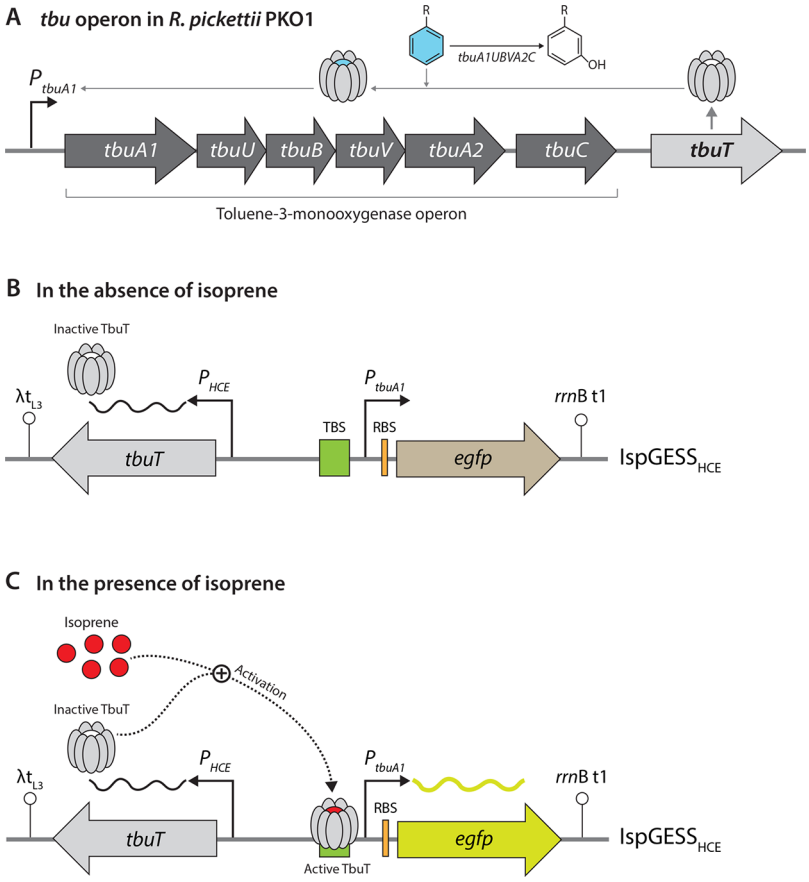

Figure 1. Schematic view of a genetically encoded isoprene biosensor, IspGESS. (A) Regulation of the *tbu* operon (*tbuA1UBVA2C* encoding toluene-3-monooxygenase) by TbuT in the *R. pickettii* PKO1 chromosome. The operon is positively regulated by TbuT in the presence of toluene or benzene and is transcribed from the P_{tbuA1} promoter. (B) Organization of IspGESS. The genes (*tbuT*, *egfp*), promoters (P_{HCE}, P_{tbuA1}), and terminators (*rrn*B t1, λt_{L3}) are indicated by thick arrows, bent arrows, and stem loops, respectively. The TbuT-binding site (TBS) and ribosome-binding site (RBS) are represented by light green and orange boxes, respectively. *tbuT* of *R. pickettii* PKO1 was expressed by P_{HCE} promoter, and the P_{tbuA1} promoter regulated the expression of *egfp*. TbuT proteins are inactive in the absence of their effectors. (C) Proposed mechanism for the IspGESS. TbuT binding to TBS, which is located upstream of the P_{tbuA1} promoter, is triggered by isoprene (small red circles), which leads to the expression of the reporter gene, *egfp*.

of intracellular small molecules serving as molecular reporters.[18] Recently, several transcriptional regulators have been used to generate whole-cell biosensors for detecting industrially valuable chemicals. For example, LysG of *Corynebacterium glutamicum* has been used for detecting L-lysine,[19] PcaU of *Acinetobacter* sp. ADP1 for sensing 3,4-dihydroxy benzoate,[20] and QdoR of *Bacillus subtilis* and FdeR of *Herbaspirillum seropedicae* for detecting flavonoids.[21] In addition, transcriptional regulators were engineered to alter the effector specificity and subsequently enabled the recognition of their non-natural ligands including mevalonate, triacetic acid lactone, ectoine, and lactulose.[22−25]

With these biosensors, a reporter gene that typically encodes a fluorescent protein is expressed from a transcriptional regulator-responsive promoter. Upon induction of the transcription factor by a metabolite of interest, the reporter protein generates a fluorescent signal proportional to the metabolite concentration. The main benefit for employing the fluorescent reporter proteins is that fluorescence-activated cell sorting (FACS) can be used for high-throughput screening at the single-cell level, which markedly reduces the time and labor required for analyzing a large number of variant enzymes or cells.[19] To use FACS for high-throughput screening, individual cells produce a sortable fluorescent protein under the control of a transcription regulator. Thus, the primary bottleneck for creating the genetically encoded biosensor is identifying a transcriptional regulator sensitive to the metabolite of interest. If the metabolites of interest are industrial chemicals, the host microorganisms usually lack cognate regulators that can sense them. However, if we introduce an orthogonal regulatory mechanism into the host from other bacteria, we can create novel metabolic biosensors with the desired input−output relationship. For instance, transcriptional regulators recognizing plant secondary metabolites were identified in plant symbiotic bacteria.[26]

Here, we present a genetically encoded biosensor for isoprene, a key intermediate in the terpenoid pathway. We developed a whole-cell biosensor that enables monitoring of the intracellular isoprene concentration in single bacterial cells and demonstrated that the specific fluorescence of isoprene producing *E. coli* corresponded with the amount of produced bioisoprene. We imported the transcriptional regulator TbuT from *Ralstonia pickettii* PKO1 (which regulates the toluene−benzene utilization [*tbu*] pathway) and its promoter P_{tbuA1}.[27,28] The *tbu* operon encodes a toluene-3-monooxygenase, and the *tbu* pathway involves a broad range of effectors such as monosubstituted benzene derivatives, ethylbenzene, trichloroethylene,[29] and isoprene.[30] When we introduced TbuT into

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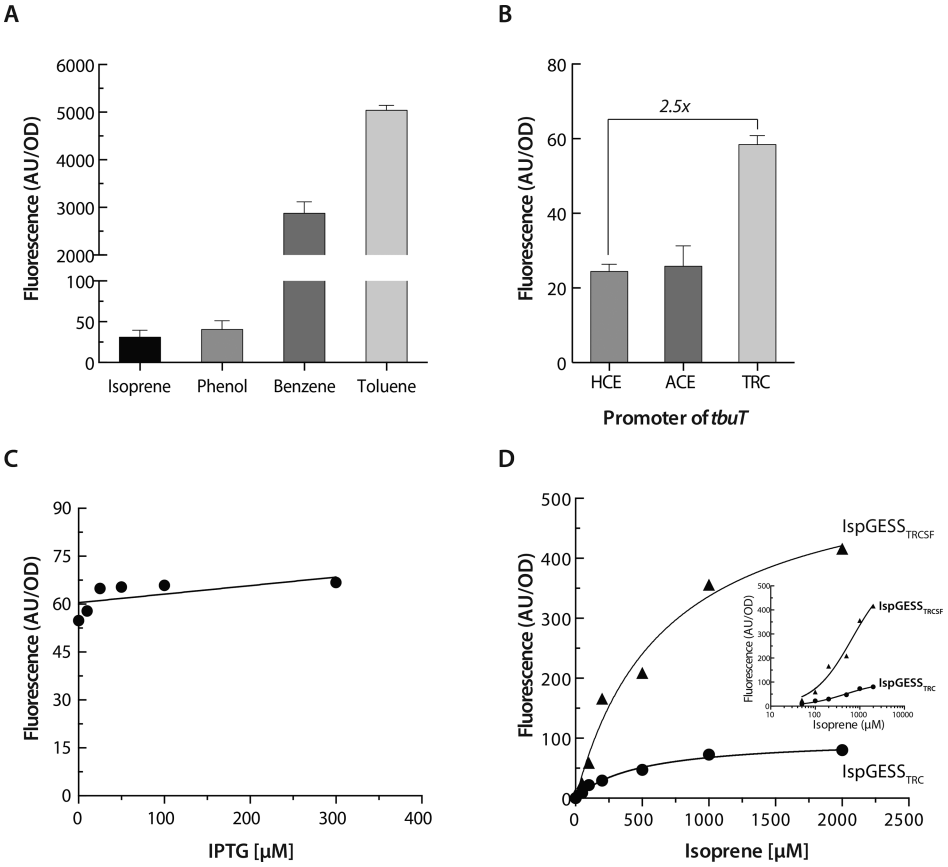

Figure 2. Characterization and improvement of the sensitivity of the IspGESS isoprene biosensor. (A) Effector specificity of IspGESS. The specific fluorescence of *E. coli* cells containing an IspGESS$_{TRC}$ plasmid was determined after 30 h of cultivation in the presence of the indicated effectors (0.1 mM each). Toluene and benzene were used as controls since they are known as natural ligands of TbuT of *R. pickettii* PKO1. (B) Effects of the promoter on TbuT expression. A strong constitutive P_{HCE} promoter and two inducible promoters, P_{ACE} and P_{TRC}, which are induced by acetate and IPTG, respectively, were examined. (C) Effects of the IPTG concentration. After we substituted the P_{HCE} promoter with the P_{TRC} promoter to control TbuT expression, we probed the effects of IPTG concentration on the specific fluorescence of IspGESS$_{TRC}$. (D) The fluorescence intensity was measured as a function of the isoprene concentration. The specific fluorescence of IspGESS$_{TRC}$ and IspGESS$_{TRCSF}$ (IspGESS$_{TRC}$ with a sfGFP reporter protein) was measured after 30 h of cultivation in the presence of various concentrations of isoprene. The inset plot is shown on a $\log_{10}$ scale to show the linear range of isoprene concentrations and IspGESS$_{TRC}$ responses. The error bars are shown as ± standard deviation (n = 3).

E. coli cells to generate an isoprene-sensing biosensor, the regulon performed poorly outside of its native host. We thus improved its sensitivity by a T7 RNA polymerase-mediated transcriptional cascade that serves to amplify the reporter gene-expression capacity to maximize cell responses. We then assessed its signal-amplification activity by FACS, which successfully identified the highest isoprene producer out of a small pool of isoprene-producing *E. coli* strains. This isoprene biosensor will be applied to identify active enzymes in a high-throughput manner to explore enzyme genotypes competent at producing isoprene.

RESULTS AND DISCUSSION

Design and Construction of Isoprene Biosensor. To develop a genetically encoded biosensor responding to isoprene (named IspGESS), a literature search was performed because we presumed that bacteria naturally exposed to isoprene would have possible transcriptional regulators that can be adopted as isoprene biosensors. Indeed, a XylR-type transcriptional regulator of *Ralstonia pickettii* PKO1, TbuT, was previously shown to be activated upon binding to isoprene.[30] TbuT binds its natural ligands, toluene and benzene, to promote their degradation. In the presence of toluene or benzene, TbuT activates the expression of the *tbu* operon (*tbuA1UBVA2C*), which is the first enzyme catalyzing the detoxification pathway of toluene and benzene (Figure 1A).[29] We took advantage of this native regulatory system to create an isoprene biosensor capable of converting the intracellular isoprene concentration into an optical fluorescence output. We first generated a single plasmid containing both the TbuT transcriptional regulator and an *egfp* fluorescent reporter, which is under the control of the *tbuT* responsive promoter, P_{tbuA1} (Figure 1B). The P_{tbuA1} promoter fragment (346 bp), which was located at 40 bp upstream of the *egfp* reporter gene, contained the −24 and −12 promoter sequences for TbuT binding and 60 bp of *tbuA1*.[31] In the initial constructed biosensor (named IspGESS$_{HCE}$), *tbuT* was expressed by a strong constitutive promoter P_{HCE} in the direction opposite to the transcription of *egfp* (Figure 1B). The expression of *egfp* was expected to be regulated by isoprene alone, inside the cell. The λt_{L3} and *rrn*B t1 terminators were used for terminating the transcription of *tbuT* and *egfp*, respectively. The IspGESS$_{HCE}$ plasmid was derived from a commonly used high-copy plasmid, which employs a pUC origin of replication. TbuT proteins are presumably nonfunctional in the absence of their effectors, whereas TbuT binds to

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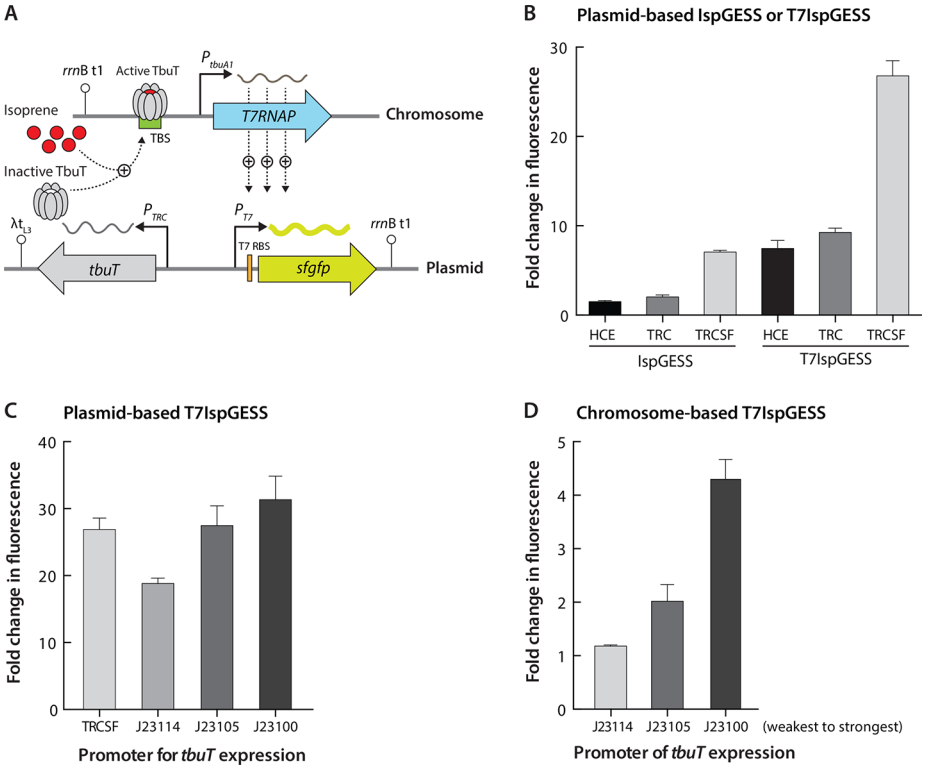

Figure 3. Transcriptional amplification cascade with the T7IspGESS. (A) Schematic representation of T7IspGESS. Isoprene activates TbuT derived from T7IspGESS, which then trans-activates the P_{tbuA1} promoter and expression of the T7 RNA polymerase (T7RNAP) in the *E. coli* chromosome. In turn, T7RNAP activates expression of *sfgfp* in the T7IspGESS plasmid, generating a fluorescent signal. (B) Comparison of the fold change in fluorescence obtained with the T7IspGESS plasmid *versus* that obtained with the IspGESS plasmid. The T7IspGESS showed several-fold increases in fluorescence with respect to the corresponding IspGESS. (C) Systematic tuning of TbuT expression in the T7IspGESS plasmid using constitutive BioBrick promoters with previously determined relative activities. Improved fold-changes in fluorescence were observed with the J23105 and J23100 promoters. (D) Fold-changes in fluorescence with the T7IspGESS plasmid integrated into the *E. coli* chromosome. Chromosome-based T7IspGESS$_{J23100SF}$ exhibited the highest fold-change in fluorescence and was further used for endogenous isoprene monitoring in engineered *E. coli* cells. The fluorescence of plasmid-borne IspGESS or T7IspGESS was measured in the presence of 1 mM isoprene for 6 h of cultivation, whereas that of chromosomal T7IspGESS was determined after 12 h of cultivation in the presence of 1 mM isoprene. Fold change in GFP fluorescence was calculated as fold change $= \frac{\mathrm{RFU}_{xv} / \mathrm{OD}_{xv}}{\mathrm{RFU}_{\mathrm{null}} / \mathrm{OD}_{\mathrm{null}}}$, where RFU and OD are relative fluorescence units and optical density values at 600 nm, respectively.[33] The subscript *xv* designates the tested cells harboring the IspGESS or T7IspGESS (in the presence of 1 mM isoprene, whereas null indicates a control cell containing the IspGESS or T7IspGESS (in the absence of isoprene). The error bars represent the standard deviation (n = 3).

the P_{tbuA1} promoter and leads to *egfp* expression in the presence of isoprene (Figure 1C). In this work, TbuT served as the transcriptional regulator to detect the presence of an isoprene and to induce the expression of a fluorescent reporter protein. Gogerty *et al.* previously constructed a TbuT-based isobutene biosensor in *E. coli*, in which the LacZ (*β*-galactosidase)-encoding gene was under the transcriptional control of P_{tbuA1} promoter region in a medium-copy number plasmid.[32] The TbuT-based isobutene biosensor was also induced by isoprene (1.3-fold induction), which is relatively smaller than those by isobutene (1.64-fold) and toluene (3.58-fold).

Characterization of the Isoprene Biosensor. To monitor *in vivo* isoprene production in *E. coli*, we harnessed the functionality of the naturally evolved regulator, TbuT, which belongs to the NtrC family of transcriptional regulators and binds to isoprene.[30] By incorporating TbuT and its cis-regulatory element P_{tbuA1} promoter, we generated biosensors (IspGESS) to recognize exogenous and endogenous isoprene (Figure 1).

Next, we examined the performance of the transcriptional regulator, TbuT, of the constructed isoprene biosensor in a heterologous host. *E. coli* DH5*α* cells transformed with the IspGESS$_{HCE}$ construct were grown in M9 minimal medium supplemented with different concentrations of various effectors. Responses of *E. coli*-IspGESS$_{HCE}$ cells to various amounts of four effectors (toluene, benzene, phenol, and isoprene) were determined (Figure S1). The transformant cells clearly responded to induction by natural effector molecules (0.1 mM toluene and benzene) and showed a 250- and 120-fold fluorescence increase relative to the uninduced control, respectively (Figure 2A). As expected, the signal-to-noise ratio for 0.1 mM isoprene and phenol was poor with this genetic construct, as measured by fluorometry and flow cytometry, which makes it difficult to correctly estimate the ratio between maximum induced signal and uninduced signal (Figure 2A). Thus, we enhanced *egfp* expression level by substituting the constitutive P_{HCE} promoter, which regulates *tbuT* expression, with the inducible P_{ACE} or P_{TRC} promoter, which is induced by acetate or isopropyl *β*-D-1-thiogalactopyranoside (IPTG), respectively. Replacing the P_{HCE} promoter of IspGESS$_{HCE}$ with the P_{TRC} promoter (IspGESS$_{TRC}$) increased *egfp* expression more than 2.5-fold in the presence of 0.1 mM IPTG and isoprene, whereas the P_{ACE} promoter showed no

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increase in *egfp* expression (Figure 2B). In addition to isoprene inside the cells, *egfp* expression from the IspGESS$_{TRC}$ plasmid is also regulated by IPTG because *tbuT* expression is modulated by the P_{TRC} promoter. To test the effect of IPTG concentration, DH5α cells harboring the IspGESS$_{TRC}$ construct were grown in M9 minimal media and induced with 0.1 mM isoprene and different concentrations of IPTG. IPTG induced *egfp* expression at all concentrations examined, but the level of induction was not significant, although GFP fluorescence slightly increased when the IPTG concentration was increased from 0 to 25 μM (Figure 2C). This result indicated that the basal leakiness of the P_{TRC} promoter is sufficient to functionally express *tbuT* (Figure S2). In general, protein expression plasmids with the P_{TRC} promoter contain the plasmid-borne *lacI* (or *lacI*^{q} gene) to tightly control the expression of the target gene. However, the IspGESS$_{TRC}$ plasmid does not possess and encode its own *lacI*; thus, only the LacI protein expressed from the chromosomal *lac* operon in the *E. coli* host represses the multicopy *tbuT* gene under uninduced conditions, which may not be sufficient to prevent the leaky TbuT expression. Thus, for all subsequent experiments, we used DH5α cells harboring the IspGESS$_{TRC}$ plasmid grown in the absence of IPTG, which is not an economical inducer.

The fluorescence response of individual cells to various isoprene concentrations was examined with *E. coli*-IspGESS$_{TRC}$ cells expressing the eGFP reporter protein (Figure 2D). A linear dependence between isoprene concentration and eGFP fluorescence was observed in the range of 0.05−2 mM, and 0.05 mM isoprene was the limit of isoprene detection by *E. coli*-IspGESS$_{TRC}$ cells (inset to Figure 2D). The maximal fluorescence increase was 7.5-fold when *E. coli*-IspGESS$_{TRC}$ cells were exposed to exogenous 2 mM isoprene. We further engineered the IspGESS$_{TRC}$ plasmid by changing the reporter protein from eGFP to super folder GFP (sfGFP), resulting in the IspGESS$_{TRCSF}$ plasmid.

Improvement of the Isoprene Biosensor Using a T7RNAP-Based Amplification Cascade. We observed an increase in the fluorescence intensity per cell with *E. coli*-IspGESS$_{TRCSF}$ cultures exposed to each isoprene concentration, although the detection limits were almost the same as those of *E. coli*-IspGESS$_{TRC}$ cultures (Figure 2). To stably express IspGESS$_{TRCSF}$ in *E. coli*, we alleviated the metabolic burden, and removed the need for biosensor plasmid maintenance with antibiotics. We integrated a single copy of the plasmid-based IspGESS$_{TRCSF}$ elements into the *E. coli* DH5α chromosome. When we compared the fluorescence under the same conditions used for the plasmid-based IspGESS$_{TRCSF}$ constructs, the chromosome-based IspGESS$_{TRCSF}$ showed no fluorescence response with any of the isoprene concentrations tested (0.05−2 mM).

To create a chromosome-based isoprene biosensor that efficiently detects intracellular isoprene produced from heterologous pathways, we further improved the sensitivity of the plasmid-based IspGESS$_{TRCSF}$ construct. We amplified the promoter/cellular response to isoprene by coupling the activity of the P_{tbuA1} promoter with T7 RNA polymerase (T7RNAP) by designing a regulatory cascade (Figure 3A). In this T7RNAP-based genetic construct (named T7IspGESS), isoprene binds the TbuT, which then activates the P_{tbuA1} promoter and causes T7RNAP expression from the chromosome of *E. coli* BL21(DE3) cells. In turn, T7RNAP transactivates the T7 promoter in front of *egfp* or *sfgfp* of the biosensor plasmid (Figure 3A). The fold-changes in fluorescence were 5.0-fold, 4.5-fold, or 3.8-fold higher than those in an equivalent *E. coli* BL21(DE3) strain bearing a plasmid containing the P_{tbuA1} promoter directly fused to the IspGESS$_{HCE}$, IspGESS$_{TRC}$, or IspGESS$_{TRCSF}$ reporter gene, respectively (Figure 3B). This cascade endowed the *E. coli* strain harboring the T7IspGESS$_{TRCSF}$ plasmid with a considerable response to M9 minimal medium containing 1 mM isoprene. However, since the P_{TRC} promoter was frequently used for isoprene producing plasmids, it was necessary to use an orthogonal promoter against P_{TRC} promoter for generating the isoprene biosensor.

To this end, we adopted well-characterized BioBrick promoters to systematically tune the expression of *tbuT* and examined the effect of *tbuT*-expression levels on the improved sensitivity of the T7IspGESS$_{TRCSF}$ construct. When we substituted the P_{TRC} promoter of *tbuT* in T7IspGESS$_{TRCSF}$ constructs with constitutive promoter activity (J23114, J23105, or J23100; shown in the order of increasing activity), the J23105 and J23100 promoters (T7IspGESS$_{J23105SF}$ or T7IspGESS$_{J23100SF}$) showed higher fold change in fluorescence than did the T7IspGESS$_{TRCSF}$ construct, whereas the J23114 promoter showed less fluorescence (Figure 3C). We then integrated the T7IspGESS$_{J23114SF}$, T7IspGESS$_{J23105SF}$ or T7IspGESS$_{J23100SF}$ plasmid into *E. coli* chromosome to generate bacterial strains, each with a single copy of the T7IspGESS$_{J23114SF}$, or T7IspGESS$_{J23100SF}$ plasmid. Among them, the chromosomal T7IspGESS$_{J23100SF}$ showed the highest fold change, whereas the chromosome-based T7IspGESS$_{J23114SF}$ exhibited a small fold change (Figure 3D).

Transcriptional amplifiers of genetically encoded biosensors can enhance transcriptional signals with a large output dynamic range.[34] Our new platform of IspGESS biosensors adopted an artificial signal amplifier (T7RNAP-based transcriptional cascade) to improve the signal-to-noise ratio of the isoprene biosensor. The T7RNAP-based signal amplifier increased the dynamic range of isoprene detection. Due to the modularity and orthogonality of the T7RNAP-based signal amplification cascade, this amplifier can be applied to a variety of weak biosensors in synthetic biology and metabolic engineering. T7RNAP-based signal amplifier has been used to increase the signal output because the transcription of genes by T7RNAP is five times faster than that by *E. coli* RNAP.[35−37] T7RNAP is very specific to the T7 promoter, resulting in an even stronger promoter than the *E. coli* promoter.[38] To tune the responses of *E. coli* cells harboring T7IspGESSs to exogenous isoprene, we altered the promoter strength for TbuT using well-characterized BioBrick promoters and found optimal promoter strengths in both plasmid-based and chromosomal T7IspGESSs. The sensitivity of both plasmid-based and chromosomal T7IspGESSs increased as the constitutive promoter strength increased. Detailed characterization of the strength and copy number of promoters and the expression of transcriptional regulator play a key role in developing a sensitive biosensor.[39]

Chromosomal T7IspGESS Biosensor. Some biosensors showed biomodal or heterogeneous induction of fluorescence,[40,41] which limited their applicability to the high-throughput screening of enzymes and metabolic pathways. To probe the florescence induction pattern of the chromosomal T7IspGESS biosensor, we compared the fluorescence intensity by flow cytometry with that by fluorometer. Fluorescence of chromosome-based IspGESS$_{J23100SF}$ was

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determined by a flow cytometer in the presence of various concentrations of isoprene (0−8 mM, Figure 4). At each

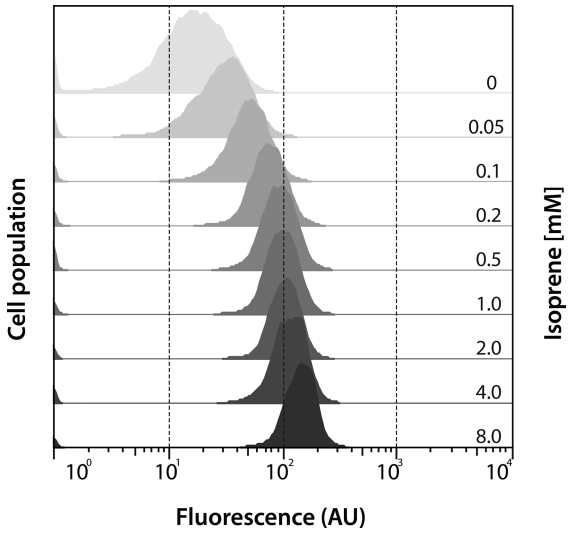

Figure 4. Specific fluorescence of chromosome-based T7IspGESS$_{J23100SF}$. Fluorescence was induced for 16 h after the addition of different concentrations of isoprene in *E. coli* cells with the T7IspGESS$_{J23100SF}$ plasmid integrated into the chromosome. Representative FACS-generated histograms from three independent experiments are shown.

isoprene concentration, most individual cells showed fluorescence in response to the isoprene concentration, indicating that the individual cell responses reflected the population-averaged behavior measured by the fluorometer. In addition, the result showed that the chromosomal T7IspGESS$_{J23100SF}$ biosensor also required at least 0.05 mM isoprene to detect isoprene and showed a linear relationship between the fluorescence and isoprene concentration at concentrations up to 8 mM (Figure 4). Previously, a biosensor using the TbuT regulator induced GFP fluorescence in the presence of 0.2 mM isoprene in *Pseudomonas fluorescens* A506 cells, but the minimal isoprene concentration for fluorescence induction was not reported.[30]

Efforts to produce isoprene in engineered *E. coli* has previously been reported,[8,9,11,42] and the best isoprene producing *E. coli* strain produced a titer of up to 123.6 g/L with 2.21 g/L/h productivity in aerobic fed-batch fermentation.[43] The produced isoprene continuously escaped from the fermenter, and the off-gas was analyzed for isoprene quantification and purification. Thus, if the isoprene production rate is 2.21 g/L/h at air flow of 1 vvm (air per culture volume per min), the off-gas isoprene level is 36.5 mg/L (541 μM). Thus, the actual isoprene concentration in the culture broth is much less than the isoprene titer in aerobic fed-batch fermentation. Therefore, the chromosome-based T7IspGESS may be suitable for analyzing and screening strains producing isoprene at this concentration.

Here, we showed the first FACS analysis coupled with a biosensor for the detection of intracellular isoprene in single bacterial cells. Previous FACS screening methods included a few targets including natural or synthetic photophores (carotenoids),[44] the antibiotic (gramicidin S),[45] or polyhydroxyalkanoates.[46] The specificity and dynamic range of a biosensor using a transcriptional regulator depend on the native biochemical properties of the chosen regulator: in the case of TbuT, toluene and benzene have been described as natural strong effectors. Thus, the intracellular detection of isoprene using the IspGESS biosensor does not necessitate secondary screening (for example by gas chromatography) to identify false-positive clones because wild-type *E. coli* cannot produce toluene or benzene.

Monitoring Enhanced Production of Bioisoprene. The primary goal of a biosensor is to screen enzymes, pathways, or cells producing a target metabolite. Thus, we sought to monitor endogenous isoprene produced in engineered *E. coli* strains with a chromosomally integrated T7IspGESS$_{J23100SF}$. Because the produced isoprene activates the TbuT transcription factor and proportionately drives sfGFP expression (Figure 3), isoprene production can potentially be monitored *in vivo* using the chromosome-based T7IspGESS biosensor.

To examine the applicability of T7IspGESS, we first introduced a plasmid expressing isoprene synthase (IspS) from *Populus trichocarpa* (pT-IspS)[47] into *E. coli* BL21(DE3) cells harboring the chromosome-based T7IspGESS$_{J23100SF}$ (*E. coli*-T7IspGESS$_{J23100SF}$) and then analyzed cells by FACS at the single cell level. Expression of IspS alone in the *E. coli*-T7IspGESS$_{J23100SF}$ strain resulted in a similar number of fluorescent cells compared with the empty plasmid, indicating that we could not detect the IspS activity within each individual cell that expressed only the IspS enzyme (Figure 5C, left). Thus, we measured the isoprene concentration produced by the *E. coli*-T7IspGESS$_{J23100SF}$ strain expressing the IspS enzyme by GC−MS, which was 5.3 μM isoprene after 24 h, whereas isoprene was not detected in the negative control strain (Figure 5C, right). This result is consistent with the sensitivity of the chromosomal T7IspGESS$_{J23100SF}$, which required at least 0.05 mM isoprene to activate the isoprene-responsive TbuT regulator.

Previously, it was shown that isoprene production increased as the amount of the added MVA increased.[7] Therefore, we introduced the pSSN12Didi plasmid,[48] which encodes 4 enzymes that convert MVA to DMAPP late in the MVA pathway (Figure 5A), into *E. coli* BL21(DE3) cells harboring the chromosome-based T7IspGESS$_{J23100SF}$ and the IspS enzyme (Figure 5B). We monitored fluorescence responses of *E. coli*-T7IspGESS$_{J23100SF}$ strain transformed with the pT-IspS and pSSN12Didi plasmid to different concentrations of MVA. The specific fluorescence emitted from transformant cells increased as the added amount of MVA increased (Figure S3). When 5 mM MVA was added to the culture broth, the *E. coli* cells produced 100 μM isoprene (Figure 5C, right), resulting in a symmetric distribution of the fluorescence with a higher fluorescent intensity, whereas the negative control with the empty plasmid showed low fluorescence, indicating that the sensing ability of the isoprene biosensor is dependent on a functional IspS and sufficient MVA. Thus, we could detect the IspS activity within each individual cell by flow cytometry.

Next, the fluorescence intensity according to different concentrations of the produced isoprene was measured using IspS enzyme and the entire MVA pathway, which converts acetyl-CoA to DMAPP (Figure 5A). We used a pTM-IspS plasmid[47] encoding the whole MVA pathway containing 7 genes catalyzing 8 metabolic steps from acetyl-CoA to isoprene, which produces isoprene from glycerol (Figure 5B). The whole MVA pathway contains seven genes; *ispS* from *P. trichocarpa*, *mvaE* and *mvaS* from *Enterococcus faecalis*, *mvaK2* and *mvaD* from *Streptococcus pneumoniae*, *mvaK1* from

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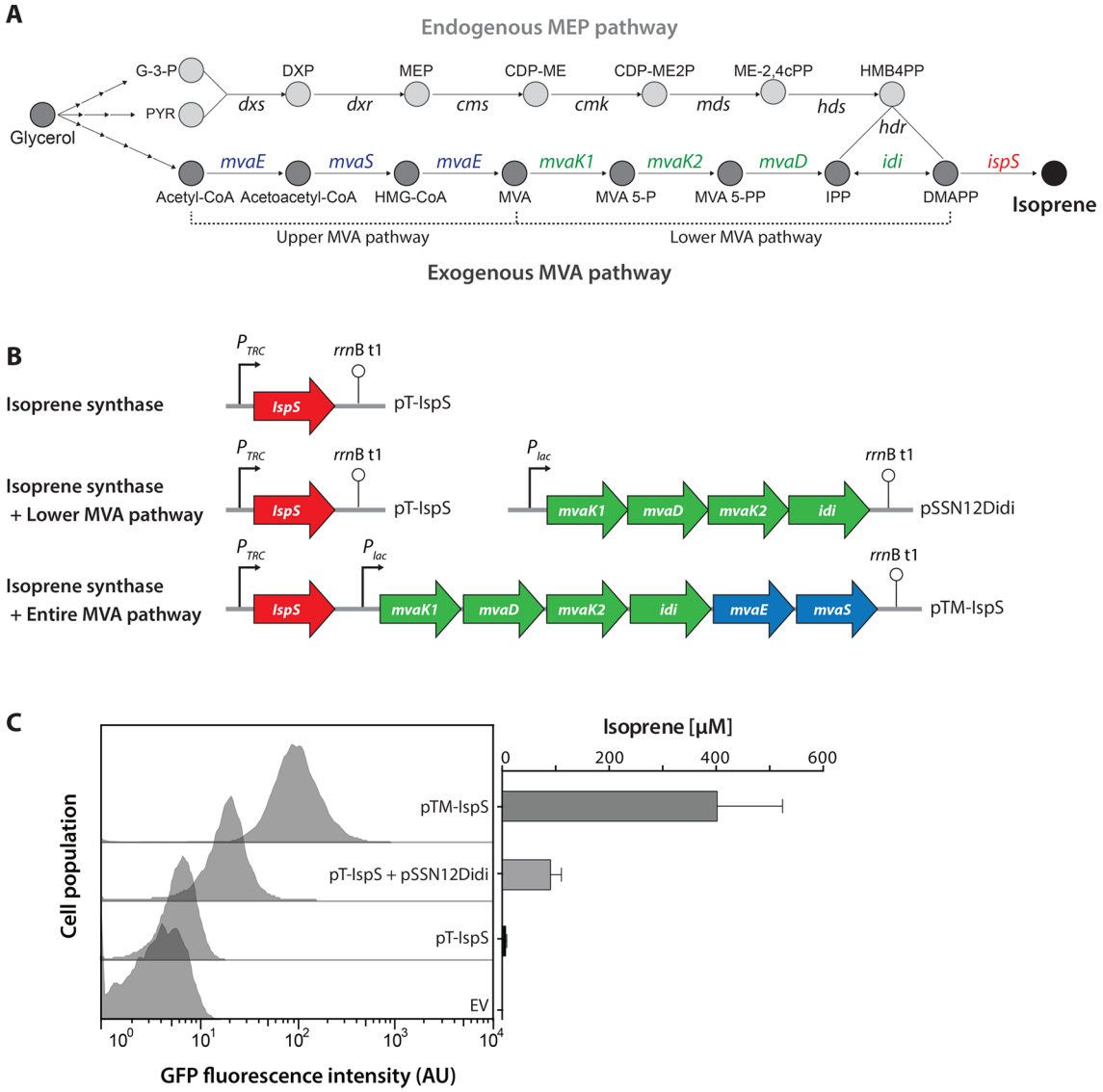

Figure 5. Monitoring endogenous isoprene production in engineered *E. coli* cells. (A) The isoprene synthetic pathway, starting from glycerol. Isoprene is produced in engineered *E. coli* cells expressing an endogenous 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway and an exogenous mevalonate (MVA) pathway. The native MEP pathway in *E. coli* consists of the following: DXS, deoxyxylulose 5-phosphate synthase; DXR, deoxyxylulose 5-phosphate reductoisomerase; CMS, 2-C-methylerythritol 4-phosphate cytidyl transferase; CMK, 4-(cytidine 5′-diphospho)-2-C-methylerythritol kinase; MDS, 2-C-methylerythritol 2,4-cyclodiphosphate synthase; HDS, (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate reductase; and HDR, hydroxymethylbutenyl diphosphate reductase. Engineered mevalonate pathway consists of the following: MvaE, acetoacetyl-CoA thiolase/3-hydroxy-3-methylglutaryl-CoA reductase; MvaS, 3-hydroxy-3-methylglutaryl-CoA synthase; MvaK1, mevalonate kinase; MvaK2, phosphomevalonate kinase; MvaD, mevalonate 5-diphosphate decarboxylase; Idi, isopentenyl diphosphate isomerase and IspS, isoprene synthase from *Populus trichocarpa*. (B) Schematic maps of plasmids used for isoprene production in *E. coli* cells. (C) Flow cytometric analyses and measurement of isoprene in *E. coli* cells harboring individual isoprene-producing plasmid by GC.

Staphylococcus aureus, and *idi* from *E. coli* (Figure 5A). We transformed the pTM-IspS plasmid into *E. coli*-T7IspGESS$_{J23100SF}$ cells for isoprene production and detection. The resulting strains were analyzed in terms of their specific fluorescence and isoprene levels after 24 h of cultivation. As the endogenous expression of the synthetic MVA pathway increased, isoprene production by the engineered *E. coli* increased (up to 400 μM), together with fluorescence (Figure 5C), indicating that the specific fluorescence of the chromosomal T7IspGESS biosensor was dependent on the isoprene produced in the engineered *E. coli* cells. A number of recent reports showed promising results for the high-throughput screening of enzymes involved in metabolic production pathways.[19,23,25,49] In the present study, we proved the use of isoprene biosensors in determining intracellular isoprene concentrations. Hence, the isoprene biosensor can be applied for high-throughput screening of isoprene synthases from metagenome or mutant libraries.[50]

Finally, to test whether the developed isoprene biosensor could function in multiple bacterial species, we constructed an IspGESS plasmid, pSEVA221-IspGESS$_{TRCSF}$ (low copy number, RK2 ori, Kan^R) and introduced it into *Pseudomonas putida* KT2440 or S12. When cultivated in M9 minimal medium supplemented with glucose, the *P. putida* KT2440 and S12 biosensor strains exhibited an increased fluorescent signal when the exogenous isoprene concentration was increased to 2

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Table 1. Bacterial Strains and Plasmids Used in This Study

strains or plasmids	description	reference
Bacterial strains		
E. coli DH5α	*F−, Φ80lacZ·ΔM15·f(lacZYA-argF)U169 deoR recA1 endA1 hsdR17(rk−, mk+) phoA supE44 thi-1 gyrA96 relA1*	Thermo Fisher Scientific
E. coli BL21(DE3)	*E. coli* B *F− dcm ompT hsdSB(rB− mB−) gal lon λ(DE3 [lacI lac*UV5-T7 gene 1 *ind*1 *sam*7 *nin*5])	Thermo Fisher Scientific
E. coli BL21(DE3) P_{tbuA1}:T7RNAP	BL21(DE3) derivatives where T7 RNA polymerase is under the control of P_{tbuA1} promoter	This study
E. coli-T7IspGESS$_{J23100SF}$	BL21(DE3)P_{tbuA1}:T7RNAP derivatives where T7IspGESS$_{J23100SF}$ was integrated into chromosomal *bglA* locus	This study
E. coli-T7IspGESS$_{J23105SF}$	BL21(DE3)P_{tbuA1}:T7RNAP derivatives where T7IspGESS$_{J23105SF}$ was integrated into chromosomal *bglA* locus	This study
E. coli-T7IspGESS$_{J23114SF}$	BL21(DE3)P_{tbuA1}:T7RNAP derivatives where T7IspGESS$_{J23114SF}$ was integrated into chromosomal *bglA* locus	This study
P. putida KT2440	Plasmid-free *P. putida* mt-2 derivative	51
P. putida S12	*Pseudomonas* strain able to degrade styrene	52
Plasmids		
pKD46	P_{araB} λ-Red expressing plasmid, Amp^R	53
pREDI	P_{araB} λ-Red, P_{rhaB} I-SceI endonuclease expressing plasmid, Amp^R	54
pKD3/I-SceI	pKD3, I-SceI endonuclease recognition site introduced at both side of the Cam resistance gene, Cam^R, Amp^R	55
pSEVA221	Low copy number vector, *ori*(RK2), Kan^R	56
pSEVA331	Medium copy number vector, *ori*(pBBR1), Cam^R	56
IspGESS$_{HCE}$	P_{HCE}:TbuT, P_{tbuA1}:eGFP expressing plasmid, Amp^R	This study
IspGESS$_{ACE}$	P_{ACE}:TbuT, P_{tbuA1}:eGFP expressing plasmid, Amp^R	This study
IspGESS$_{TRC}$	P_{TRC}:TbuT, P_{tbuA1}:eGFP expressing plasmid, Amp^R	This study
IspGESS$_{TRCSF}$	P_{TRC}:TbuT, P_{tbuA1}:sfGFP expressing plasmid, Amp^R	This study
T7IspGESS$_{HCE}$	P_{HCE}:TbuT, P_{T7}:eGFP expressing plasmid, Amp^R	This study
T7IspGESS$_{TRC}$	P_{TRC}:TbuT, P_{T7}:eGFP expressing plasmid, Amp^R	This study
T7IspGESS$_{TRCSF}$	P_{TRC}:TbuT, P_{T7}:sfGFP expressing plasmid, Amp^R	This study
T7IspGESS$_{J23114SF}$	P_{J23114}:TbuT, P_{T7}:sfGFP expressing plasmid, Amp^R	This study
T7IspGESS$_{J23105SF}$	P_{J23105}:TbuT, P_{T7}:sfGFP expressing plasmid, Amp^R	This study
T7IspGESS$_{J23100SF}$	P_{J23100}:TbuT, P_{T7}:sfGFP expressing plasmid, Amp^R	This study
pTrc99A	P_{TRC}, pBR322 ori, Amp^R	GE Healthcare Life Sciences
pT-IspS	pTrc99A with *E. coli*-codon optimized *Populus trichocarpa ispS*	47
pSSN12Didi	pSTV28 with *mvaK1*, *mvaK2*, and *mvaD* from *Streptococcus pneumoniae*, and *idi* from *E. coli*	48
pTM-Isp	pT-Isp containing *mvaK1* of *S. aureus*, *mvaD* and *mvaK2* of *S. pneumoniae*, *idi* of *E. coli*, *mvaE* and *mvaS*, of *E. faecalis*	47
pSEVA221-IspGESS$_{TRCSF}$	pSEVA221 containing IspGESS$_{TRCSF}$ cassette	This study
pSEVA331-IspGESS$_{TRCSF}$	pSEVA331 containing IspGESS$_{TRCSF}$ cassette	This study

mM, resulting in 12.8- and 20.5-fold dynamic ranges, respectively (Figure S4). We also generated a pSEVA331-IspGESS$_{TRCSF}$ plasmid (medium copy number, pBBR1 ori, Cam^R) to examine the effects of increased TbuT regulator expression on the fluorescence response. *P. putida* KT2440 cells harboring the pSEVA331-IspGESS$_{TRCSF}$ plasmid also showed a dynamic response to up to 2 mM exogenous isoprene, and the fold increase (24.3-fold) was higher than that in the KT2440 strain with pSEVA221-IspGESS$_{TRCSF}$ (Figure S5). Therefore, our isoprene biosensor functioned in other bacterial species.

CONCLUSIONS

In this study, we have generated and improved IspGESS biosensors for the key isoprenoid pathway intermediate, isoprene, which has been exploited as a commodity chemical.[4] The transcription factor, TbuT, was adopted from a soil bacteria *R. pickettii* PKO1 and heterologously expressed in *E. coli*. The initial IspGESS biosensor showed a very weak response to exogenous isoprene, whereas it exhibited either a very strong response or high sensitivity toward natural benzyl family molecules, like toluene and benzene. After we tuned the IspGESS biosensors and amplified their fluorescence signals using a T7RNAP-mediated cascade system (T7IspGESS biosensors), we could detect both exogenous and endogenous isoprene by fluorescence signal that is dependent on the isoprene concentration. This isoprene biosensor will be applied to identify novel enzymes and pathways involved in isoprene production for improving known isoprene synthases or optimizing isoprene production and thereby help to develop microbial cell factories for isoprene production.

MATERIALS AND METHODS

Bacterial Strains and Reagents. The strains and plasmids used in this study are presented in Table 1. The *E. coli* strain DH5α was used for cloning, and the strains DH5α and BL21(DE3) were used for biosensor experiments. SOC medium (20 g/L tryptone, 5 g/L yeast extract, 0.5 g/L NaCl, 2.4 g/L $MgSO_4$, 186 mg/L KCl, and 4 g/L glucose) was used for growth recovery after transformation. Luria−Bertani (LB) medium (10 g/L tryptone, 5 g/L yeast extract, and 5 g/L NaCl) and M9 minimal medium (6.78 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 0.5 g/L NaCl, 1 g/L NH_4Cl, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, and 0.5 μg/mL thiamine) supplemented with an

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additional carbon source (4 g/L glucose) were used for bacterial cultivation and biosensor development, respectively. M9 minimal medium containing 20 g/L glycerol was used for isoprene production. High fidelity KOD-Plus-Neo polymerase (Toyobo, Osaka, Japan) was used for polymerase chain reaction (PCR) experiments, following the manufacturer's protocol. All restriction enzymes, T4 DNA ligase, T4 polynucleotide kinase, and the Gibson Assembly Master Mix were purchased from New England BioLabs (Ipswich, MA, USA).

Plasmid Construction. Sanger sequencing and/or restriction analysis was used to confirm correct coding and assembly in all recombinant plasmids constructed in this study.

IspGESS$_{HCE}$, IspGESS$_{ACE}$, and IspGESS$_{TRC}$. The oligonucleotides used in this study are presented in Table S1. *tbuT* and *tbuA1* promoter were amplified from the genomic DNA of *R. pickettii* PKO1 (ATCC 27511) using target-specific primers (TbuT-F and TbuT-R for *tbuT* and PtbuA1-F and PtbuA1-R for the *tbuA1* promoter). The P_{HCE} promoter for *tbuT* expression was amplified using primers HCE-F and HCE-R, with the pHCE-IIB plasmid serving as the template. Using three PCR-amplified DNA fragments, overlap-extension PCR was performed to generate P_{HCE}-TbuT-P_{tbuA1} flanked with the *Eco*RI restriction enzyme site. After digestion with *Eco*RI, the P_{HCE}-TbuT-P_{tbuA1} was ligated into the pGESSv1 plasmid[50] that was predigested with *Eco*RI, resulting in the IspGESS$_{HCE}$ plasmid. To replace the *tbuT* promoter of IspGESS$_{HCE}$ plasmid with the P_{ACE} or P_{TRC} promoter, the P_{ACE} or P_{TRC} promoter was synthesized by commercial vendor (Bioneer, Daejeon, South Korea) and ligated into the IspGESS$_{HCE}$ plasmid digested with *Hin*dIII and *Bam*HI, which generated IspGESS$_{ACE}$ or IspGESS$_{TRC}$, respectively.

T7IspGESS$_{TRC}$, T7IspGESS$_{HCE}$, T7IspGESS$_{TRCSF}$, T7IspGESS$_{J23105SF}$, and T7IspGESS$_{J23114SF}$. The IspGESS$_{TRC}$ plasmid was digested with *Bam*HI and *Kpn*I to remove the *tbuA1* promoter of enhanced GFP (eGFP). The remaining fragment was ligated with the T7 promoter (constructed by annealing the T7PA-F and T7PA-R primers), which resulted in T7IspGESS$_{TRC}$. The T7IspGESS$_{HCE}$ plasmid was constructed as described above using the IspGESS$_{HCE}$ plasmid instead of the IspGESS$_{TRC}$ plasmid. To construct a T7IspGESS$_{TRCSF}$ plasmid, the whole region (excluding eGFP) was amplified from T7IspGESS$_{TRC}$ with the GibV-F and GibV-R primers. Superfolder GFP (sfGFP)[57] was synthesized (Bioneer, Daejeon, South Korea) and amplified with the GibI-F and GibI-R primers. The two amplified fragments were assembled *via* the Gibson Assembly method.[58] To create the T7IspGESS$_{J2310SSF}$ plasmid, the T7IspGESS$_{TRCSF}$ plasmid was digested with NcoI and ClaI to remove the P_{TRC} promoter of *tbuT*. The fragment was ligated with the J23105 promoter (constructed by annealing the J23105-F and J23105-R primers). T7IspGESS$_{J23114SF}$ plasmid was constructed as described above using the J23114-F and J23114-R primers.

pSEVA221-IspGESS$_{TRCSF}$ and pSEVA331-IspGESS$_{TRCSF}$. The regions containing *tbuT*- and *sfgfp*-expression cassettes were amplified from the IspGESS$_{TRCSF}$ plasmid with the SeGeV-F and SeGeV-R primers. The pSEVA221 plasmid backbone containing an RK2 origin of replication and kanamycin-resistance gene was amplified from the pSEVA221 plasmid[59] using the SeGeI-F and SeGeI-R primers. Both amplified fragments were assembled *via* the Gibson Assembly Method. The pSEVA331-IspGESS$_{TRCSF}$ plasmid was constructed as described above using the pSEVA331 plasmid.[59]

BL21(DE3) P_{tbuA1}:T7RNAP Strain Construction. We swapped the *lac*UV5 promoter of T7 RNA polymerase (T7RNAP) with the *tbuA1* promoter using a markerless-deletion method, based on λ Red-mediated homologous recombination.[53,60] First, we amplified the antibiotic-resistance cassette with homology arms from pKD3/I-SceI and transformed the PCR product into BL21(DE3) harboring pKD46 and swapped the *lac*UV5 promoter region of BL21(DE3) with the aid of λ Red helper plasmid pKD46. After curing the pKD46 helper plasmid by growth at 37 °C, we transformed a pREDI plasmid encoding the recombinant proteins, L-rhamnose-inducible I-SceI endonuclease and L-arabinose inducible λ Red. For the second recombination, we synthesized a *tbuA1* promoter containing an upstream *rrnB* t1 terminator to prevent read-through transcription from the neighboring region (Bioneer, Daejeon, South Korea). After PCR amplification of the *rrnB* t1 terminator and *tbuA1* promoter region with homology arms, we transformed the PCR fragment into the modified BL21(DE3) strain harboring pREDI, replaced the upstream region of T7RNAP with a *tbuA1* promoter by λ Red-mediated recombination, and selected a modified strain by I-SceI-mediated double strand break killing.

BL21(DE3) P_{tbuA1}:T7RNAP T7IspGESS Strain Construction. We integrated three T7IspGESS modules (T7IspGESS$_{J23114SF}$, T7IspGESS$_{J2310SSF}$, and T7IspGESS$_{J23100SF}$) individually into the *bglA* locus using a markerless-deletion method. First, we modified the T7IspGESS$_{TRC}$ plasmid containing the chloramphenicol-resistance gene flanked by I-SceI-recognition sites, which was used as a landing pad. To construct the landing pad plasmid, we amplified the backbone region from IspGESS$_{TRC}$ with the GibGESSR-F2 and GibGESSL-R2 primers. The chloramphenicol-resistance cassette was amplified from pKD3/I-SceI with the GibIsceI-F2 and GibIsceI-R2 primers. Both amplified fragments were assembled *via* the Gibson Assembly method. Then, we amplified the chloramphenicol-resistance cassette from the landing pad plasmid with homology arms and transformed the PCR product into BL21(DE3)P_{tbuA1}:T7RNAP cells harboring pKD46 and integrated the landing pad into the *bglA* locus with the aid of λ Red helper plasmid pKD46. After curing the pKD46 by growth at 37 °C, we the transformed cells with the pREDI plasmid. For the second recombination, we amplified the various T7IspGESS modules from T7IspGESS plasmids with homology arms. Then, we transformed the PCR fragment into the BL21(DE3)P_{tbuA1}:T7RNAP strain with landing pad and pREDI, introduced the landing pad into the T7IspGESS module by λ Red-mediated recombination, and selected the modified strain by I-SceI-mediated double strand break killing.

Culture Conditions and Isoprene Supplementation. Saturated isoprene solutions were prepared by mixing equal volumes of isoprene with culture medium overnight in a 27 °C incubator with gentle shaking. The saturated isoprene aliquots in TB were transferred to fresh TB medium to prepare the desired isoprene concentration based on Henry's law. The isoprene concentrations were also independently verified by a gas chromatography (GC) system equipped with a flame ionization detector with an HP-5 column. Single *E. coli* colonies harboring a biosensor plasmid were individually inoculated into M9 minimal medium containing appropriate antibiotics and cultured at 30 °C, 200 rpm overnight. Then, the cultures were inoculated at OD_{600} = 0.05 into fresh 3 mL M9 minimal medium supplemented with appropriate antibiotics in a 10 mL serum bottle (Wheaton Scientific Products,

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Millville, NJ). To prevent isoprene evaporation, the serum bottles, microtubes, and tips were prechilled, and the subsequent experiment was performed in a cold room (4 °C). After chilling, the saturated isoprene solution was added to the culture medium with capillary piston (Gilson, Villiers Le Bel, France), and rapidly sealed with stopper and aluminum seal (Wheaton Scientific Products). The sealed bottles with inoculated cells were cultured at 30 °C and 200 rpm.

Isoprene Determination. To measure isoprene production, 1 mL of gas sample from the headspace of sealed cultures was analyzed as described earlier[61] using a gas chromatography (GC) system equipped with a flame ionization detector with an HP-5 column (30 m × 0.320 mm × 0.25 μm) at a flow rate of 1 mL/min. The starting temperature of the oven was maintained at 40 °C for 3 min; the temperature was increased at 10 °C/min to 100 °C, held at 100 °C for 3 min, increased to 200 °C at 30 °C/min, and then again held at 200 °C for 1 min. Isoprene (Sigma−Aldrich, St. Louis, MO, USA) was used as an external standard to quantify isoprene production. Three biological replicates were used to measure isoprene production to ensure reproducibility.

Fluorescence Assay. Fluorescence and OD_{600} measurements were conducted with the Victor X multilabel plate reader (PerkinElmer, Waltham, MA, USA) using black-walled 96-well polystyrene plates after dilution into the linear range of the detector. Single-cell fluorescence analysis was performed using a FACSCalibur instrument (BD Bioscience, Franklin Lakes, NJ, USA). Three biological replicates were used for all fluorescence assays. In all cases, fluorescence was normalized using OD_{600} values, and the background fluorescence due to the buffer was subtracted from all measurements. Fold change in GFP fluorescence was calculated as fold change $= \frac{RFU_{xv} / OD_{xv}}{RFU_{null} / OD_{null}}$, where RFU and OD are relative fluorescence units and optical density values at 600 nm, respectively.[33] The subscript *xv* designates the tested cells harboring the pIspGESS or pT7IspGESS plasmid (in the presence of 0.1 mM isoprene), whereas null indicates a control cell containing the pIspGESS or pT7IspGESS plasmid (in the absence of isoprene).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.8b00164.

Supplemental figures, tables and references (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

*E-mail: dhlee@kribb.re.kr. Tel: + 82 42 879 8225. Fax: +82 42 860 4489.

*E-mail: sglee@kribb.re.kr. Tel: + 82 42 860 4373. Fax: +82 42 860 4489.

ORCID

Dae-Hee Lee: 0000-0002-0423-9057

Present Addresses

[‖]Samsung Biologics, Incheon 21987, Republic of Korea.

[⊥]Department of Microbiology, School of Medicine, Chungnam National University, Daejeon 35015, Republic of Korea.

Author Contributions

D.L. and S.L. conceived and supervised the project; S.K.K., S.K., B.H., S.W. and E.R. performed the experiments; S.K. and H.K. participated in analyzing the results. S.K.K., D.L., and S.L. wrote the manuscript.

Notes

The authors declare no competing financial interest.

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