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CRISPR interference-guided balancing of a biosynthetic mevalonate pathway increases terpenoid production

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ABSTRACT

Methods for simple and efficient regulation of metabolic pathway genes are essential for maximizing product titers and conversion yields, and for minimizing the metabolic burden caused by heterologous expression of multiple genes often in the operon context. Clustered regularly interspaced short palindromic repeats (CRISPR) interference (CRISPRi) is emerging as a promising tool for transcriptional modulation. In this study, we developed a regulatable CRISPRi system for fine-tuning biosynthetic pathways and thus directing carbon flux toward target product synthesis. By exploiting engineered *Escherichia coli* harboring a biosynthetic mevalonate (MVA) pathway and plant-derived terpenoid synthases, the CRISPRi system successfully modulated the expression of all the MVA pathway genes in the context of operon and blocked the transcription of the acetoacetyl-CoA thiolase enzyme that catalyzes the first step in the MVA pathway. This CRISPRi-guided balancing of expression of MVA pathway genes led to enhanced production of (-)-α-bisabolol (C15) and lycopene (C40) and alleviation of cell growth inhibition that may be caused by expression of multiple enzymes or production of toxic intermediate metabolites in the MVA pathway. Coupling CRISPRi to cell growth by regulating an endogenous essential gene (*ispA*) increased isoprene (C5) production. The regulatable CRISPRi system proved to be a robust platform for systematic modulation of biosynthetic and endogenous gene expression, and can be used to tune biosynthetic metabolic pathways. Its application can enable the development of microbial 'smart cell' factories that can produce other industrially valuable products in the future.

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1. Introduction

Tunable and balanced expression of heterologous genes is essential for high productivity, product titer, and conversion yield during metabolic pathway engineering of microorganisms (Alonso-Gutierrez et al., 2015; Du et al., 2012; Zelcbuch et al., 2013). During the transfer of genes involved in complex biosynthetic pathways to heterologous hosts, the native regulation of metabolic flux through these pathways is often lost. This causes imbalances in the metabolites produced by the associated exogenous pathways and subsequently limits product titers and yields. Furthermore, overproduction of multiple biosynthetic enzymes and intermediate metabolites, which are sometimes toxic to heterologous hosts, results in growth inhibition or genetic mutations that decrease the yield and productivity (Zhang et al., 2012). However, many efforts in the microbial production of valuable products are mainly focused on building metabolic pathways to obtain the products. Several regulated gene expression tools have been used in metabolic engineering to improve the output of biosynthetic pathways (Na et al., 2013; Wang et al., 2009). Recently, the clustered regularly interspaced short palindromic repeat (CRISPR) interference (CRISPRi) system from bacteria has been repurposed in order to determine how changes in gene expression programs lead to diverse cellular phenotypes. The CRISPRi system is also being increasingly used to engineer more sophisticated custom cell lines for biotechnological applications

Abbreviations: CRISPR, Clustered regularly interspaced short palindromic repeats; CRISPRi, Clustered regularly interspaced short palindromic repeats interference; MVA, mevalonate; sgRNA, single guide RNA; dCas9, inactive Cas9; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; MEP, methylerythritol 4-phosphate; DXP, 1-deoxy-D-xylulose 5-phosphate; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; LB, Luria-Bertani; TB, terrific broth; PCR, polymerase chain reaction; UTR, untranslated region; T7RNAP, T7 RNA polymerase; sfGFP, superfolder GFP; RBS, ribosome binding site; IPTG, isopropyl β-D-1-thiogalactopyranoside; PBS, phosphate buffered saline; RT-PCR, real-time PCR; GC, gas chromatography; FID, flame ionization detector; CAT, chloramphenicol acetyl transferase; PAM, protospacer adjacent motif; IspA, farnesyl diphosphate synthase

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(Cress et al., 2015; Nielsen and Voigt, 2014).

Initially, CRISPR attracted significant attention as a genome-editing tool because of the orthogonality of CRISPR RNA-guided Cas9 endonucleases and the simplicity associated with the design of the single guide RNA (sgRNA) that is required for gene-specific targeting (Cong et al., 2013). As such, the use of CRISPR/Cas9-mediated genome editing has been reported in many organisms including bacteria, plants, mammals, and fungi (Jakociunas et al., 2015; Jiang et al., 2013; Shan et al., 2013; Wang et al., 2013). The original CRISPR approach was used to completely and irreversibly knockout gene expression. In contrast, the CRISPRi system harnesses a catalytically inactive Cas9 (dCas9) protein, which lacks endonuclease activity but retains DNA binding function and can be targeted to multiple loci simultaneously by using locus-specific Cas9-binding sgRNAs (Qi et al., 2013). A particularly interesting application of the CRISPRi system is in the rewiring and regulating diverse cellular metabolic networks by repression of sequence-specific target genes (Gilbert et al., 2014; Zhao et al., 2014). Indeed, transcription-level control of complex multigene programs using the CRISPRi system has recently been demonstrated (Gilbert et al., 2014; Qi et al., 2013). In metabolic engineering efforts, the CRISPRi system has been used to adjust and increase biosynthesis pathway fluxes of a biodegradable material polyhydroxyalkanoate and a plant flavonoid naringenin (Cress et al., 2015; Lv et al., 2015; Wu et al., 2015).

Terpenoids have been used for a long time in flavorings, fragrances, pharmaceuticals, insecticides, and drugs (Breitmaier, 2006; Connolly and Hill, 1991). With the growing need for large-scale production of terpenoid drugs (Dahl et al., 2013) and the emerging use of terpenoids for biofuel production (Zhang et al., 2011), significant advancement has been made in the designing and building of terpenoid biosynthetic pathways in microbes (Dahl et al., 2013; George et al., 2015; Martin et al., 2003; Paddon and Keasling, 2014). All terpenoids, comprising the most diverse class of natural products are derived from the universal five-carbon building block, isopentenyl diphosphate (IPP), and its allylic isomer, dimethylallyl diphosphate (DMAPP). These intermediates are synthesized in two different pathways: the mevalonate (MVA) pathway and non-mevalonate pathway (the latter is also referred to as the methylerythritol 4-phosphate (MEP)/1-deoxy-D-xylulose 5-phosphate (DXP) pathway) (Kirby and Keasling, 2009; Kuzuyama, 2002; Liu et al., 2013). Engineering both these pathways to enhance terpenoid production in microbial hosts would impose metabolic burdens on the cells because of the competition between the use of the pathways for cell growth and terpenoid production. Thus, balancing the expression levels of the associated enzymes would be required, which is a time-consuming and laborious task (Ajikumar et al., 2010; Nowroozi et al., 2014; Pfleger et al., 2006). In a previous study, balanced expression of pathway enzymes was required to maximize flux towards terpenoid synthesis while minimizing the accumulation of toxic intermediates of the MVA pathway, such as IPP and 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) (Kizer et al., 2008; Martin et al., 2003). Moreover, numerous targets for the microbial production of terpenoids, such as genes essential for cell growth, are yet to be identified owing to the inconvenience of conventional gene knockout methods.

Here, we presented a regulatable CRISPRi system to fine-tune the expression of heterologous pathway genes and thus to enhance biosynthetic pathway productivity. Our model pathways were those that produce the smallest terpene (isoprene, C5), sesquiterpene alcohol ((-)-α-bisabolol, C15), and carotenoid (lycopene, C40). To accomplish this, we first constructed a single L-rhamnose-inducible CRISPR plasmid that harbors both a catalytically inactive dCas9 and an sgRNA cassette. Next, genes in the MVA pathway that are essential to cell growth were repressed using the CRISPRi system to identify individual target genes that could balance the expression of the MVA pathway genes. Furthermore, the efficiency of repression of the target genes was tuned to strike a balance between terpenoid production and cell growth. Finally, we targeted multiple gene sites, including promoter and internal regions, to repress gene expression and to achieve high terpenoid yield. Thus, we provide a transformative tool that can be used in metabolic engineering.

2. Materials and methods

2.1. Bacterial strains and reagents

The bacterial strain used for cloning was *Escherichia coli* DH5α and the strains DH5α and BL21(DE3) strains were used for terpenoid production. Luria-Bertani (LB) medium (10 g/L tryptone, 5 g/L yeast extract, and 5 g/L NaCl) and terrific broth (TB) medium containing glycerol (12 g/L enzymatic casein digest, 24 g/L yeast extract, 9.4 g/L K_2HPO_4, 2.2 g/L KH_2PO_4, and 3.5% (w/v) glycerol) were used for bacterial cultivation and terpenoid production, respectively. Ampicillin (100 μg/mL), kanamycin (25 μg/mL), or chloramphenicol (34 μg/mL) was added to ensure plasmid maintenance. High-fidelity KOD-Plus-Neo polymerase (Toyobo, Osaka, Japan) was used for polymerase chain reaction (PCR). All the restriction enzymes, T4 DNA ligase, T4 polynucleotide kinase, and Gibson Assembly Master Mix were purchased from New England Biolabs (Ipswich, USA).

2.2. CRISPRi plasmid construction

The primers and plasmids used in this study are summarized in Supplementary Tables S1 and S2, respectively. To construct a dCas9 expression plasmid, we amplified the dCas9 gene using the pdCas9-bacteria plasmid (Addgene plasmid 44,249) as a template. The PCR-amplified dCas9 gene was then assembled with a linear DNA fragment prepared from *Nde*I and *Nar*I digestion of a pAR-mlacI plasmid (Yoon et al., 2014) by using an EZ-Fusion Cloning Kit (Enzynomics, Daejeon, Korea), yielding the plasmid pAR-dCas9, containing an L-rhamnose inducible $rhaP_{BAD}$ promoter upstream of the dCas9 gene. To express both the dCas9 gene and an sgRNA in a single plasmid, we synthesized the sgRNA cassette (Bioneer, Daejeon, Korea) with sequences targeting the 5′ untranslated region (UTR) of T7 RNA polymerase (T7RNAP), amplified the product, and assembled it with the linear DNA fragment prepared from *Nar*I digestion of a pAR-dCas9 plasmid by using the EZ-Fusion Cloning Kit to obtain plasmid pACCRi. To construct a CRISPRi system that would be expressed at a low copy number, we amplified the dCas9 gene along with an L-rhamnose promoter and a sgRNA cassette from pACCRi, and used the Gibson Assembly Method to incorporate it into a backbone fragment that was amplified from a pSEVA221 plasmid (Silva-Rocha et al., 2013), thus generating the plasmid pSECRi. For multiplex CRISPRi, we constructed sgRNA arrays using restriction enzymes; we removed the *Age*I and *Xma*I sites within the *rhaR* and *rhaS* genes using McriV-F/McriV-R or McriI-F/McriI-R primers that have synonymous changes on the *Age*I/*Xma*I sites. Using these two primer pairs, we then amplified two fragments from a pSECRi plasmid and joined them using the Gibson Assembly Method. Each sgRNA in multiplex CRISPRi arrays had its own promoter (J23119).

2.3. Reporter plasmid construction

A pREGFP1 previously named as a pMW7(GFP) (Kwon et al., 2015) was used for a single-targeting CRISPRi of the T7 RNAP in *E. coli* BL21(DE3). For a dual-targeting CRISPRi of T7 RNAP and GFP,

we constructed a pREGFP2 plasmid by inserting sgRNA(T1) binding site between T7 promoter and GFP in the pREGFP1. To construct a gene reporter plasmid, we replaced T7 promoter of pREGFP1 with a constitutive BioBrick BBa_J23100 promoter. We then amplified the entire region except for the T7 promoter from pREGFP1, which contains a BBa_J23100 promoter sequence. After amplification, we ligated the linear amplicon using T4 DNA ligase and T4 polynucleotide kinase, thus generating the pREGFP3 plasmid. For constructing operon reporter plasmids, we replaced the *mvaD* gene of the MVA pathway with a superfolder GFP (sfGFP) gene (Pedelacq et al., 2006). We then amplified a backbone fragment (excluding the *mvaD* gene) from a pTM-ISP plasmid, which was created in this study for isoprene production. We also amplified an sfGFP gene from a pK7-sfGFP plasmid (Lee et al., 2016) and joined the two fragments using the Gibson Assembly Method. Using this construct, we then amplified a P*lac-mvaK1-sfGFP* cassette and assembled it with a backbone fragment amplified from a pSEVA131 plasmid (Silva-Rocha et al., 2013), which yield the plasmid pREGFP4. To construct a pREGFP5 plasmid, we replaced the *mvaS* gene of the MVA pathway with the sfGFP gene. We then amplified a backbone fragment (excluding the *mvaS* gene) from the pTM-ISP plasmid, and the sfGFP gene from the pK7-sfGFP plasmid, respectively, and connected the two fragments by Gibson Assembly. Using this construct, we amplified a P*lac-mvaK1-mvaD-mvaK2-idi-mvaE-sfGFP* cassette and assembled it with the backbone fragment amplified from the pSEVA131 plasmid.

For the internal promoter test, we deleted a *lac* promoter from the pREGFP4 plasmid, which yielded the plasmid pREGFP4Δlac. To construct the control plasmid, we amplified the sfGFP gene from a pREGFP4 plasmid and assembled it with a backbone fragment amplified from a pREGFP4 plasmid by Gibson Assembly, thus generating a pREGFP4ΔMVA plasmid containing only the sfGFP gene and its 5′UTR region without a *lac* promoter and MVA pathway genes. Similarly, we deleted a *lac* promoter from the pREGFP5 plasmid, which yielded the plasmid pREGFP5Δlac. For the control plasmid, we amplified the sfGFP gene and assembled it with another fragment amplified from the pREGFP5 plasmid by Gibson Assembly, which yielded a pREGFP5ΔMVA plasmid, containing only the sfGFP gene and its 5′UTR region without the MVA pathway genes.

2.4. Terpenoid production plasmid construction

For constructing a lycopene production plasmid, we amplified a *crtEIB* operon from a pTrcAKpGalAZpTrcEBIY plasmid (Lee et al., 2013) and assembled another fragment amplified from a pT-ISP plasmid (unpublished), which yielded the plasmid pT-CRT. A pTM-CRT plasmid was constructed as follows: A pT-CRT plasmid was digested with *Xba*I and treated with shrimp alkaline phosphatase (Takara Bio, Shiga, Japan) to prevent self-ligation. A cassette encoding all the MVA pathway genes was amplified from a pSMvL$_7$-MvU$_M$ plasmid (Yang et al., 2016) and digested with *Xba*I. The two fragments were then ligated using T4 DNA ligase. For constructing various *mvaE* ribosome binding site (RBS) variants, we amplified the entire region except for a RBS in the *mvaE* gene from a pTM-CRT plasmid and then ligated the linear amplicon using T4 DNA ligase and T4 polynucleotide kinase, which yielded the plasmid pTM-CRT(RBS A). Other *mvaE* RBS variants (RBS B–E) were constructed in a similar manner. To construct an isoprene production plasmid, we digested a pT-ISP plasmid (unpublished) with *Xba*I and treated it with shrimp alkaline phosphatase. Furthermore, a pTM-CRT plasmid was digested with *Xba*I, and the fragments corresponding to the entire MVA pathway genes were gel-purified (Promega, Madison, USA). The two fragments were then ligated using T4 DNA ligase, which yielded the plasmid pTM-ISP. In order to construct a (-)-α-bisabolol production plasmid, we digested the pT-BBS plasmid (unpublished) with *Xba*I, and treated it with shrimp alkaline phosphatase. Furthermore, a pTM-CRT plasmid was digested with *Xba*I and the fragments corresponding to all the genes in the entire MVA pathway genes were gel-purified (Promega, Madison, USA). The two fragments were then ligated using T4 DNA ligase, which yielded the pTM-BBS plasmid. In all the cases, Sanger sequencing was used to verify the direction of the MVA pathway genes.

2.5. Fluorescence assay

All the binding sites of the sgRNAs used for the CRISPRi system are listed in Supplementary Table S3. To assay the repression efficiency of the T7 expression system, *E. coli* BL21(DE3) cells were grown in LB medium at 37 °C overnight and then transferred to flask cultures supplemented with various concentrations of L-rhamnose ranging from 0 to 1 mM. The cells were then induced for 3 h with 0.7 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG) at optical density at 600 nm (OD_{600}) of 0.3–0.4 for endogenous T7RNAP gene expression and subjected to fluorescence measurement. *E. coli* cells harboring a reporter plasmid and a CRISPRi plasmid for MVA pathway regulation were grown in LB medium at 37 °C for 8 h. For dCas9 protein induction, 1 mM L-rhamnose was added to the culture medium. The culture broth was washed and resuspended with phosphate buffered saline (PBS). Fluorescence and OD_{600} measurements were conducted with the Victor X multi-label plate reader (PerkinElmer, Waltham, MA, USA) using black-walled 96-well polystyrene plates after dilution into the linear range of the detector. In all the cases, fluorescence was normalized using OD_{600}, and repression was calculated relative to the expression in a control strain possessing the identical reporter and the CRISPRi plasmid without L-rhamnose. A single cell fluorescence analysis was performed using FACSCalibur (BD Bioscience, Franklin Lakes, NJ, USA) and time-course monitoring of fluorescence was performed using an Infinite 200 PRO microplate reader (Tecan, Männedorf, Switzerland). Three biological replicates were used for all the reporter assays.

2.6. Determination of ispA gene expression levels

Total RNA was isolated from the bacterial culture by using the RNeasy Protect Bacteria Mini Kit (Qiagen, Valencia, CA, USA) and the cDNA was synthesized using the ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo, Osaka, Japan). Using 16S rRNA as the internal control, real-time PCR (RT-PCR) for mRNA analysis was performed using the CFX Connect™ Real-Time PCR Detection System (BioRad, Hercules, CA, USA) with iQ™ SYBR® Green Supermix (BioRad, Hercules, CA, USA). All the samples were prepared with three parallel groups to obtain ΔCt values from the outputs of the RT-PCR. The primers used for the RT-PCR analysis are also listed in Table S1. The relative gene expression was quantified using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.7. Quantification of terpenoid products

Intracellular lycopene was extracted from 0.5 mL of bacterial culture at the end of the cultivation period. The cell pellet was washed once with 1 mL of distilled water and then extracted in 1 mL of acetone at 55 °C for 10 min at 700 rpm in a thermomixer (Eppendorf, Hamburg, Germany) under reduced light conditions (Yoon et al., 2006). The lycopene content in the supernatant of the acetone extracts (obtained by centrifugation at 14,500*g* for 10 min) was quantified by measuring absorbance at 475 nm, and the concentration was calculated by comparison with a standard curve of lycopene (Sigma-Aldrich, St. Louis, MO, USA). In the case of (-)-α-bisabolol, *n*-dodecane overlaid in the bacterial culture was

recovered by centrifugation (14,500*g* for 3 min), and the amount of (-)-α-bisabolol extracted in *n*-dodecane was determined using a gas chromatography (GC) system equipped with a flame ionization detector (FID) 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 60 °C for 2 min; this was increased at the rate of 5 °C/min to 200 °C, held at 200 °C for 2 min, increased at the rate of 50 °C/min to 300 °C, and then again held at 300 °C for 5 min (-)-α-Bisabolol (Sigma-Aldrich, St. Louis, MO, USA) was used to generate a standard curve. To measure isoprene concentration, 0.5 mL of the headspace of the sealed serum bottle used for the cultivation of the engineered *E. coli* was directly injected into the GC system equipped with the FID and the 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; this was increased at 10 °C/min to 100 °C, held at 100 °C for 3 min, increased at the rate of 30 °C/min to 200 °C, and then again held at 200 °C for 1 min. Isoprene (Sigma-Aldrich, St. Louis, MO, USA) was used as an external standard for the quantification of isoprene production. Three biological replicates were used for measuring terpenoid production to ensure reproducibility.

3. Results

3.1. Establishment of a regulatable CRISPRi system

To establish a tunable bacterial CRISPRi system, we first created a single CRISPRi plasmid harboring both a catalytically inactive dCas9 from *Streptococcus pyogenes* (Qi et al., 2013) and an sgRNA cassette under the control of an L-rhamnose inducible promoter ($rhaP_{BAD}$) and a strong constitutive BBa_J23119 promoter, respectively, to yield the CRISPRi plasmid, pACCRi (Fig. 1A). The pACCRi plasmid was derived from a pACYC plasmid with chloramphenicol resistance and p15A origin (Fig. S1). To examine the capability of the established CRISPRi system for transcriptional repression in *E.*

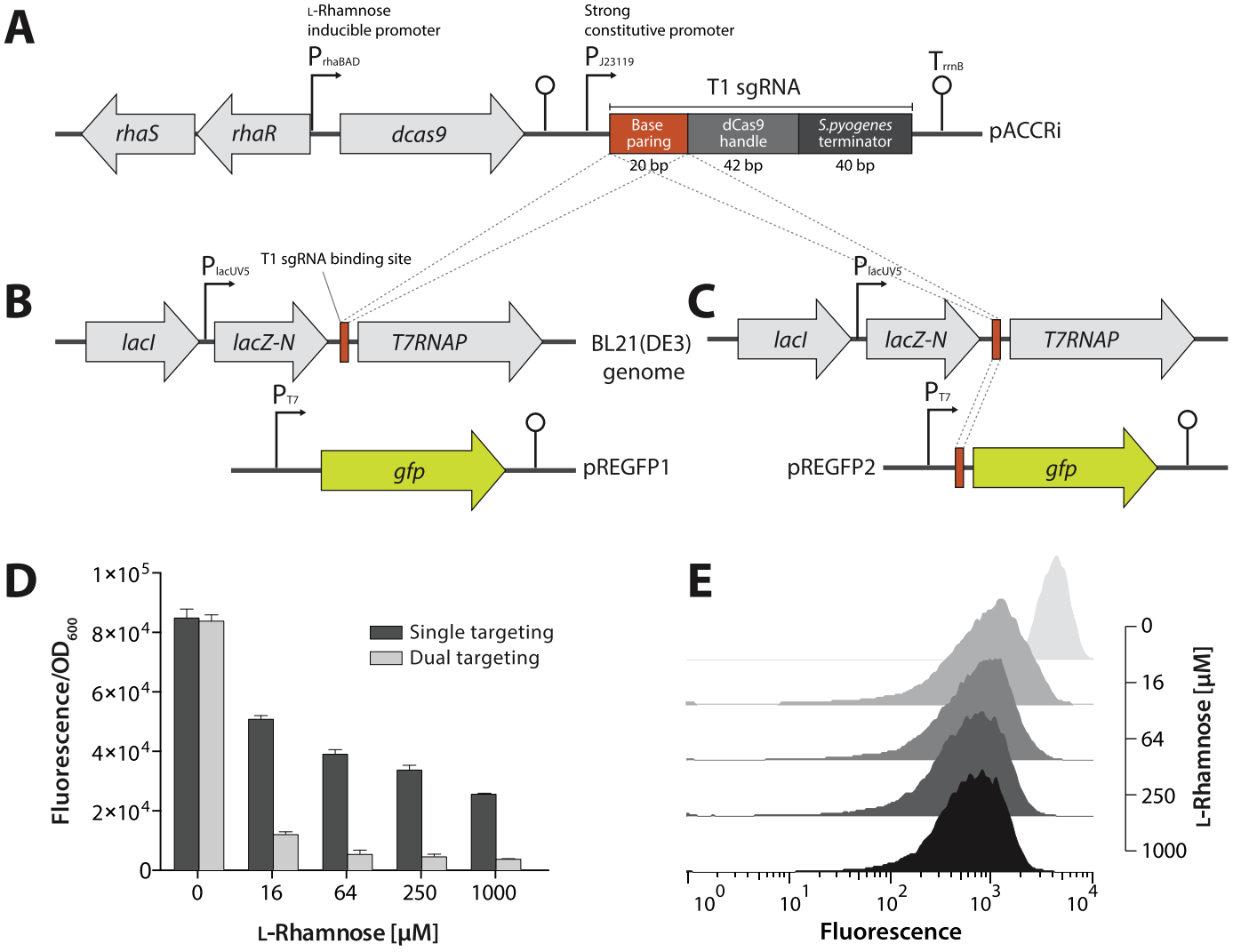

Fig. 1. Construction of the regulatable CRISPRi and reporter system for controlling endogenous and exogenous gene expression. (A) The CRISPRi system consisted of an L-rhamnose-inducible dCas9 protein and a designed sgRNA chimera encompassing a J23119 constitutive promoter, base-pairing nucleotides for specific target DNA sequence binding (20 bp), a 42-nt dCas9-binding handle, and a 40-nt transcription terminator derived from *Streptomyces pyogenes* was orthogonal to the *E. coli* system (Jinek et al., 2012; Qi et al., 2013). (B) dCas9-mediated repression of an endogenous gene, where the chromosomal T7 RNA polymerase (T7RNAP) gene of *E. coli* BL21(DE3) was repressed by targeting its 5′ UTR. Note that repression of the expression of the T7RNAP gene reduced GFP protein expression in *E. coli* BL21(DE3) cells. (C) dCas9-mediated repression of endogenous and exogenous genes, where the chromosomal T7RNAP gene of *E. coli* BL21(DE3) and plasmid-encoded GFP gene in a reporter plasmid (pREGFP2) were both repressed by targeting the 5′ UTR of them. The 20-nt complementary region for T7RNAP gene binding was inserted upstream of the GFP gene in pREGFP2. (D) CRISPRi-based repression by single targeting (T7RNAP) or dual targeting (T7RNAP and GFP) in *E. coli* BL21(DE3) cells. (E) Repression of plasmid-borne GFP gene using CRISPRi by targeting only the chromosomal T7RNAP gene in *E. coli* BL21(DE3) cells at the single cell level using flow cytometry.

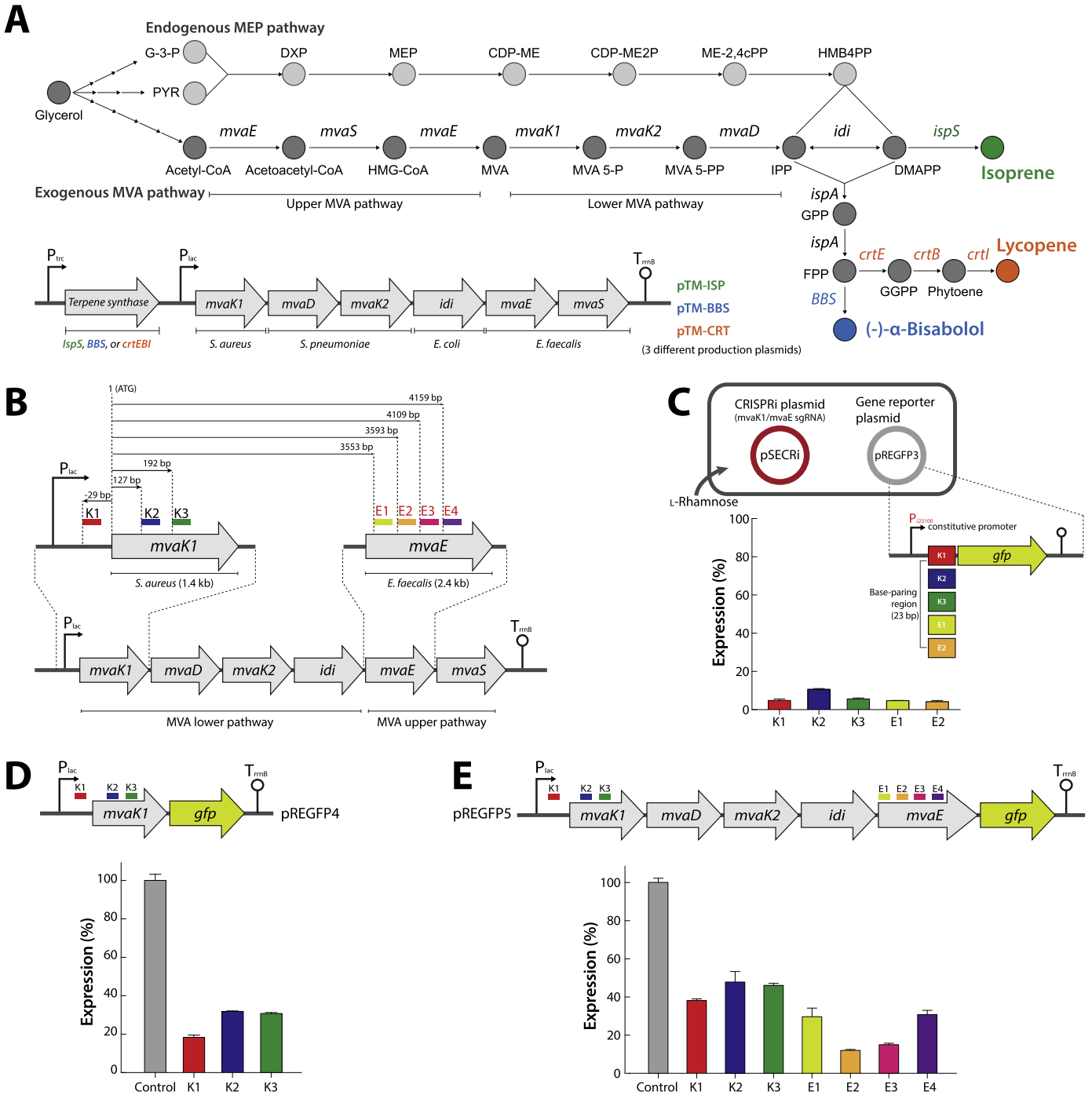

Fig. 2. Design and construction of the CRISPRi system for controlling the expression of MVA pathway genes. (A) Schematic representation of terpenoid production pathways and plasmids. Top: terpenoid production pathways: endogenous *E. coli* 2-C-methyl-D-erythritol 4-phosphate (MEP) and exogenous mevalonate (MVA) pathway. The engineered MVA pathway consists of seven enzymes: MvaE, a dual function enzyme, acetoacetyl-CoA thiolase and 3-hydroxy-3-methylglutaryl-CoA reductase of *Enterococcus faecalis*; MvaS, 3-hydroxy-3-methylglutaryl- CoA synthase of *E. faecalis*; MvaK1, mevalonate kinase of *Staphylococcus aureus*; MvaK2, phosphomevalonate kinase of *Streptomyces pneumoniae*; MvaD, mevalonate 5- diphosphate decarboxylase of *S. pneumoniae*; Idi, isopentenyl diphosphate isomerase of *E. coli*; and IspA, geranyl diphosphate synthase or farnesyl diphosphate synthase of *E. coli*; BBS, (-)-α-bisabolol synthase of German camomile (Son et al., 2014); IspS, isoprene synthase of *Populus trichocarpa* (Calfapietra et al., 2007); and *crtEIB* operon derived from metagenome (Lee et al., 2013). Bottom: An operon encoding the entire MVA pathway and terpenoid synthase genes. Three different plasmids, pTM-ISP, pTM-BBS and pTM-CRT were used for production of isoprene, (-)-α-bisabolol, and lycopene, respectively. (B) Design of seven sgRNAs blocking transcription initiation (K1–K3) and elongation (E1–E4). The K1–K3 sgRNAs block transcription initiation of all the MVA pathway genes and E1-E4 silence the activity of MvaE, which is required for the first step of the MVA biosynthetic pathway. (C) A synthetic fluorescence-based gene reporter system containing a GFP-coding gene was introduced into *E. coli*. Each sgRNAs (K2, K3, E1, or E2) that bind to the non-template DNA strand was coexpressed with the dCas9 protein, and their effects on GFP expression were measured using the in vivo fluorescence assay. The base-pairing region contains a PAM domain (5′-NGG-3′). (D, E) For the operon reporter assay, two reporter plasmids (pREGFP4 or pREGFP5) were constructed by assembling the GFP gene of the pK7-sfGFP plasmid downstream of the *mvaK1* or *mvaE* gene of the pTM-ISP plasmid. Their repression efficiency was determined using the in vivo fluorescence assay.

coli, we then generated a fluorescence-based reporter plasmid (pREGFP1) harboring a GFP (Fig. 1B). As a T7 promoter-based gene expression gives a very high expression level relative to endogenous genes (Studier and Moffatt, 1986), we tried to measure the repression efficiency of the CRISPRi system from this commonly used high-strength promoter. First, we conducted single-target repression by introducing a sgRNA(T1) targeting the endogenous chromosomal T7RNAP gene of *E. coli* BL21(DE3) into the pACCRi plasmid (Fig. 1B). L-rhamnose could repress the expression of the plasmid-borne GFP gene at all concentrations ranging from 16 to 1000 μM in a dose-dependent manner, i.e., as L-rhamnose concentration increased, GFP repression increased (Fig. 1D). Single cell analysis of single-target repression using flow cytometry revealed that the dose-dependent repression was homogenous (Fig. 1E). It is important to mention here that upon IPTG induction, the T7RNAP gene was expressed and began transcription of GFP gene on the pREGFP1 plasmid, resulting in turn in the expression of the GFP gene under the control of the T7 promoter. Although endogenous T7RNAP expression was successfully repressed, T7RNAP could still amplify the GFP gene expression in the high copy plasmid pREGFP1. Thus, in order to further repress the expression of the GFP gene, dual targeting repression was carried out using a pREGFP2 reporter plasmid containing a sgRNA(T1) binding site upstream of a GFP gene in the pREGFP1 plasmid (Fig. 1C). Instead of designing a new sgRNA targeting the GFP gene, we just inserted the sgRNA(T1) binding sequence into the 5′ UTR of the GFP gene because the repression capability of sgRNA(T1) had already been confirmed by single targeting repression (Fig. 2C). As expected, repression of GFP expression upon dual targeting of the chromosomal T7RNAP and plasmid-encoded GFP gene increased in a dose-dependent manner following addition of L-rhamnose and was significantly more effective than targeting T7RNAP alone (Fig. 1D). Dual targeting repression showed overall silencing of up to 27-fold (7- to 27-fold repression), whereas single targeting repression demonstrated little effect of up to 3.9-fold (1.7- to 3.9-fold repression, Fig. 1D).

3.2. MVA pathway regulation using CRISPRi

Considering the efficient repression mediated by the regulatable CRISPRi system, we focused on the potential of this system to modulate gene expression in the terpenoid biosynthetic pathway (Fig. 2A, top). To this end, we constructed a high copy number plasmid expressing various terpenoid synthases (isoprene synthase, (-)-α-bisabolol synthase, or lycopene synthase) under the control of a *trc* promoter; the transcription of synthetic MVA pathway genes was further enhanced by addition of a *lac* promoter, which resulted in three different terpenoid production plasmids (pTM-ISP, pTM-BBS, or pTM-CRT) (Fig. 2A, bottom). As previously described, *E. coli* cells harboring MVA pathway genes produce high concentrations of IPP and DMAPP, which are the universal five-carbon building blocks for terpenoid biosynthesis (Jang et al., 2015; Yoon et al., 2009). For MVA pathway regulation using CRISPRi, we generated a new CRISPRi plasmid (pSECRi, Fig. S1) using a low copy number plasmid, pSEVA221 (Silva-Rocha et al., 2013), because it imposes a minimal metabolic burden (Blatny et al., 1997). Additionally, pSEVA221 has a broad host range RK2 replication origin that is compatible with the widely used ColE1 and p15A origins in *E. coli*. Furthermore, the pSEVA221 plasmid harbors a kanamycin resistance gene instead of the chloramphenicol acetyl transferase (CAT) gene that was used in the medium copy number pACCRi plasmid (Fig. S1). This circumvents the issue of undesired modification of metabolites, including alcohols and monoterpenes, which is associated with promiscuous CAT activity (Alonso-Gutierrez et al., 2013; Rodriguez et al., 2014). Using two CRISPRi plasmids, pACCRi and pSECRi, we conducted a gene reporter assay. To this end, we designed five kinds of sgRNAs targeting MvaK1 (K1, K2, and K3) and MvaE (E1, E2) (Fig. 2B). The K1, K2, and K3 sgRNAs were designed to repress the transcription of all the MVA pathway genes in the context of the operon, which may alleviate metabolic burden from the expression of multiple exogenous enzymes, and sgRNA(E1) and sgRNA(E2) blocked the expression of the acetoacetyl-CoA thiolase enzyme that is encoded by the *mvaE* gene and catalyzes the first step in the MVA pathway beginning with acetyl-CoA (Fig. 2A, top), which may balance the intermediate metabolites of the MVA pathway. When designing sgRNAs, we selected the non-template DNA strand binding sequence, which also has the 10-bp seed sequence with an NGG protospacer adjacent motif (PAM) that did not perfectly match the MG1655 and BL21(DE3) genome sequences. This was done in order to avoid any non-specific binding of sgRNAs to the host genome (Table S3). For a gene reporter assay, the gene reporter plasmid contains a binding site for an individual sgRNA with an NGG PAM sequence. This directs the CRISPRi activity to the strand running opposite to the direction of transcription at a site between the GFP gene and its strong constitutive BBa_J23100 promoter (Fig. 2C). The repression activity of CRISPRi was thus quantified by the reduction in GFP fluorescence. The reporter assay revealed that all the CRISPRi systems based on the pSECRi plasmid with five kinds of sgRNAs (K1, K2, K3, E1, or E2) successfully repressed GFP expression; the final GFP expression levels were 3.3–10.6% of the control values (Fig. 2C). CRISPRi (K1) exhibited the greatest repression activity (95.2%) while blocking the expression of all the MVA pathway genes, while CRISPRi (E2) was the most effective repressor (96.7%) of MvaE expression. Although both the pSECRi- and pACCRi-based CRISPRi systems were equally effective repressors of GFP expression (Fig. S2), we chose pSECRi as the regulatable CRISPRi plasmid for our subsequent experiments due to the additional advantages mentioned above.

Although a gene reporter assay can successfully measure CRISPRi binding affinity, it cannot assess its potential to block the transcription of genes expressed in the more physiological context of an operon (Qi et al., 2013), because CRISPRi-mediated transcriptional repression is inversely related to the distance from the transcription start site (Qi et al., 2013). We therefore established an operon reporter assay using operon reporter plasmids that were created by replacing the *mvaD* or *mvaS* genes of the MVA pathway with the sfGFP gene (Fig. 2D and E). For an operon reporter assay, we designed two more sgRNAs (E3, E4) that bind to the *mvaE* gene (4109 bp and 4159 bp, respectively, from the MvaK1 translation start site) (Fig. 2B). As in the gene reporter assay, all the designed sgRNAs efficiently bound to their target sites and effectively blocked operon transcription, despite the long distance (~4 kb) between the target sites and the *lac* promoter (Fig. 2D and E). CRISPRi (K1) and CRISPRi (E2) showed the best repression activity for all the MVA pathway genes (81.6% repression) and *mvaE* gene (88% repression), respectively, which was consistent with the results of the gene reporter assay (Fig. 2C). Based on these results, we selected two sgRNAs targeting the *lac* promoter (K1) and the *mvaE* gene (E2) in order to balance the expression of the whole MVA pathway gene cassette and the first step enzyme of MVA biosynthesis, respectively.

3.3. Enhancing lycopene biosynthesis

We next examined the ability of the regulatable CRISPRi system to balance the expression of lycopene biosynthetic enzymes and subsequently to enhance lycopene production in *E. coli* DH5α cells. The biosynthetic MVA pathway and lycopene synthase for this natural plant product has been reconstituted in microbes previously (Alper et al., 2005; Farmer and Liao, 2000; Hong-Jie et al., 2015; Kim et al., 2011). We hypothesized that successful tuning of

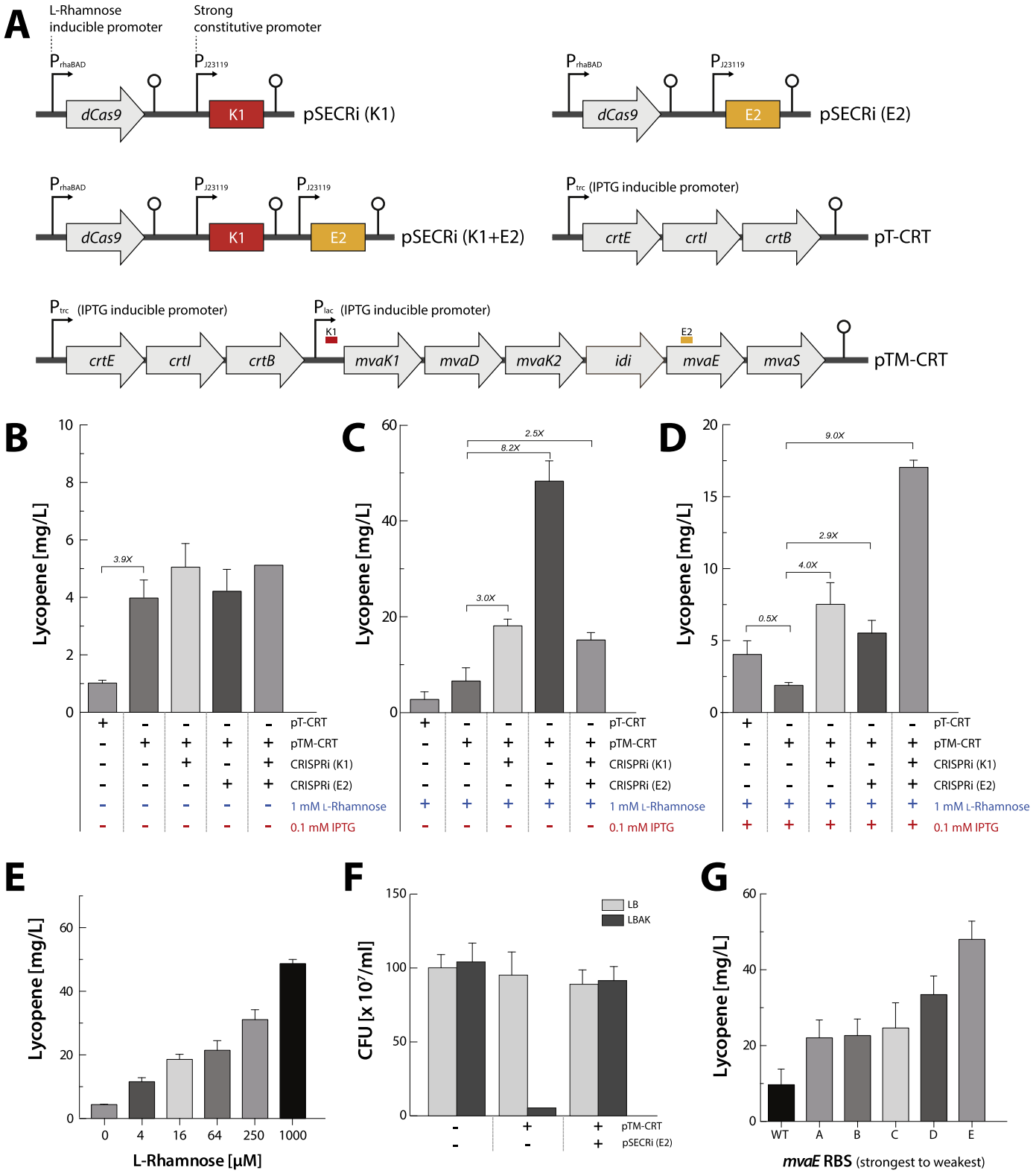

Fig. 3. Application of the CRISPRi system for enhancing lycopene production. (A) Schematic representation of the CRISPRi system and lycopene production plasmids. The CRISPRi plasmid harbors both a catalytically inactive dCas9 from *Streptococcus pyogenes* and an sgRNA cassette (K1, E2 or K1+E2) under the control of a L-rhamnose inducible promoter (rhaPBAD) and a strong constitutive BBa_J23119 promoter, respectively. The lycopene synthase (*crtEIB* operon) and MVA pathway genes are under the control of the *trc* and *lac* promoters, respectively. (B) Lycopene production in the absence of L-rhamnose and IPTG. CRISPRi (K1) and CRISPRi (E2) denotes the *E. coli* cells harboring the pSECRi (K1) and pSECRi (E2) plasmid, respectively. If CRISPRi (K1) and CRISPRi (E2) are positive, it means that the *E. coli* cells contain the pSECRi (K1+E2) plasmid. (C) In the absence of IPTG, dCas9 protein production was induced by 1 mM L-rhamnose and was coexpressed with two sgRNAs. (D) In the presence of L-rhamnose and IPTG, both CRISPRi (K1) and CRISPRi (E2) increased lycopene production, and synergized when they are coexpressed in *E. coli*. (E) L-rhamnose dose-dependent lycopene production using *E. coli* pTM-CRT with dCas9 and sgRNA(E2). (F) Enhanced lycopene production was due to the increase in plasmid stability. pT-CRT and pTM-CRT are ampicillin-resistance plasmids and CRISPRi (E2) corresponds with the presence of pSECRi (kanamycin-resistance plasmid). CFU: colony forming unit; LB: Luria-Bertani medium; LBAK: LB medium supplemented with ampicillin (100 μg/mL) and kanamycin (25 μg/mL). (G) A small set of RBS sequences was designed to verify the effect of *mvaE* repression on lycopene production. The designed RBS sequence and names were based on a previous report (Zelcbuch et al., 2013).

the cassette encoding the MVA pathway genes would lead to measurable increases in lycopene titers compared to that obtained using the unbalanced pathway. To test this notion, we created a lycopene production plasmid using the high copy number pT-CRT plasmid, which has a lycopene synthase operon (*crtEIB*) under the control of the IPTG-inducible *trc* promoter (Lee et al., 2013) (Fig. 3A). Insertion of all the genes required for MVA biosynthesis transcribed from the *lac* promoter yielded the pTM-CRT plasmid (Fig. 3A).

A previous study reported that lycopene production decreased under IPTG induction conditions (Yoon et al., 2006); therefore, we first tested the lycopene production in the absence of IPTG and L-rhamnose, which are required to induce the *crtEIB* operon of pT-CRT, the *crtEIB* operon and MVA pathway genes of pTM-CRT, and CRISPRi, respectively. In the absence of IPTG and L-rhamnose, 1.0 ± 0.1 mg/L of lycopene was produced from the pT-CRT encoding the lycopene synthase operon alone. The lycopene production increased to 3.9 ± 0.4 mg/L when the MVA pathway genes were coexpressed with *crtEIB*, and similar levels of lycopene (4.6–5.2 mg/L) were produced with single (sgRNA(K1) or sgRNA(E2)) and dual (sgRNA(K1 + E2)) targeting CRISPRi in the absence of both inducers (Fig. 3B). The result suggested that the biosynthetic MVA pathway improves lycopene production in engineered *E. coli* cells (Alper et al., 2005; Farmer and Liao, 2000; Hong-Jie et al., 2015; Kim et al., 2011) and that the constitutive expression of sgRNAs did not affect lycopene production in *E. coli*.

Next, we examined whether targeting the *lac* promoter or MvaE enzyme using the CRISPRi system could enhance the efficiency of lycopene production. To this end, we added 1 mM of L-rhamnose to induce the expression of dCas9 protein in the absence of IPTG (Fig. 3C). Repression by the corresponding sgRNA (K1 for the *lac* promoter or E2 for *mvaE*) elicited 3.0- or 8.2-fold increases in lycopene production, respectively, compared to that from the cells expressing *crtEIB* and the MVA pathway genes of pTM-CRT (Fig. 3C). Although both sgRNAs increased lycopene production, the sgRNA(E2) produced 2.7-fold more lycopene than the sgRNA(K1). However, coexpression of sgRNA(K1) and sgRNA (E2) had a suppressive effect on lycopene production compared to that observed with either sgRNA alone, thus demonstrating that the initial flux of the MVA pathway, which begins with acetyl-CoA, is more important for lycopene production under non-induced conditions. In this case, reduced acetyl-CoA flux toward lycopene biosynthesis may balance the levels of intermediate metabolites that may be toxic to lycopene-producing *E. coli*. Combining two sgRNAs showed suppressive effects on lycopene production, while still enhancing lycopene production by 2.5-fold compared with that obtained by expression of *crtEIB* and the MVA pathway genes alone (Fig. 3C). This indicates that leaky expression of the MVA pathway genes without IPTG can still impose metabolic burden on lycopene-producing *E. coli*. Indeed, the specific growth rate of *E. coli* cells harboring pTM-CRT decreased in the absence of *mvaE* repression and IPTG while the lag period increased under the same condition (Fig. S3).

In the presence of 0.1 mM IPTG and 1 mM L-rhamnose, the effects of the CRISPRi system on lycopene production varied, depending on whether lycopene synthase was induced alone or in combination with MVA pathway genes. Overexpression of lycopene synthase alone resulted in 4.03 ± 0.9 mg/L of lycopene production (Fig. 3D). However, the titer was decreased to 1.89 ± 0.2 mg/L in the *E. coli* cells expressing both *crtEIB* and the MVA pathway genes following IPTG induction, which is consistent with the results of a previous report (Yoon et al., 2006). Targeting the *lac* promoter or the *mvaE* gene using the CRISPRi system silencing expression of the entire MVA biosynthetic cassette slightly increased lycopene production to 7.50 ± 1.2 mg/L or 5.53 ± 0.8 mg/L, respectively (Fig. 3D). We observed greater combinatorial effects using the sgRNA(K1 + E2) in the presence of both inducers. When the sgRNAs were combined with CRISPRi targeting the *lac* promoter and *mvaE* gene, lycopene production increased up to 17.0 ± 0.5 mg/L, which is 9.0-fold higher than that observed with the expression of *crtEIB* and MVA pathway genes (pTM-CRT) alone, although the lycopene production (17.0 ± 0.5 mg/L) was still lower than observed when either sgRNA(K1) or sgRNA(E2) was used in the absence of IPTG (47.6 ± 5.1 mg/L). These results imply that IPTG induction reduced lycopene production due to the cellular toxicity caused by overexpression of MVA pathway genes, but dampening the transcription of MVA pathway genes using CRISPRi relieved the toxicity. Among all the conditions tested, repression of mvaE in the absence of IPTE yielded the highest concentration of lycopene (47.6 ± 5.1 mg/L) (Fig. 3C). When we conducted a combined targeting with sgRNA(E2 + E3) to repress the *mvaE*, there was no increase in lycopene production (Fig. S4).

Finally, we tested whether lycopene production was dependent on L-rhamnose concentration under the condition of *mvaE* repression and absence of IPTG. Lycopene production increased proportionally with L-rhamnose concentration when *mvaE* was targeted using the CRISPRi system (Fig. 3E); in the presence of 1 mM L-rhamnose, lycopene production increased to 47.1 ± 1.5 mg/L. Since reduced terpenoid production can be a consequence of unstable plasmid maintenance in *E. coli* (Anthony et al., 2009), we examined plasmid stability in various strains with or without CRISPRi regulation in the presence of 1 mM L-rhamnose. As expected, increased production of lycopene was related to plasmid stability, since CRISPRi regulation of *mvaE* using CRISPRi increased in up to 100% of the cells harboring a pTM-CRT plasmid compared with that in the non-CRISPRi-regulated cells (4.7%, Fig. 3F). This improvement highlights the ability of CRISPRi to impact lycopene production via simple tuning of an exogenous MVA pathway that may lead to balanced expression of the enzymes in the MVA pathway and subsequent stable maintenance of the production plasmid in *E. coli*.

Based on the MVA operon structure, it would be anticipated that CRISPRi of *mvaE* would co-repress *mvaS* expression, and that hus lycopene production may also be improved by directly repressing *mvaS* (Fig. 3A). To test this possibility, we designed two sgRNAs targeting *mvaS* (S1, S2) and confirmed their functionality using a gene reporter assay (Fig. S5). However, *mvaS* repression did not enhance lycopene production in the absence of IPTG (Fig. S5). To corroborate whether the enhanced lycopene titers were mainly dependent upon the decreased expression of *mvaE*, we constructed various plasmids that tune *mvaE* expression by RBS diversification (Zelcbuch et al., 2013). In the absence of IPTG, lowering MvaE expression by RBS engineering gradually increased lycopene production up to 48 ± 4.8 mg/L (RBS-E, Fig. 3G), which was similar to that observed with CRISPRi regulation (Fig. 3E). From these results, we concluded that the MvaE enzyme is an important regulator of the MVA pathway under non-induced conditions.

3.4. Enhancing lycopene production in suboptimal hosts

The *E. coli* BL21(DE3) strain is a major workhorse for the production of heterologous proteins and has advantages over K-12 strains that show high biomass yield and lower acetate accumulation (Phue et al., 2008; Shiloach et al., 1996; Yoon et al., 2012). However, in terms of terpenoid production, BL21(DE3) is less effective than K-12 strains (Yoon et al., 2009) despite their similar growth properties. This may be due to the toxicity associated with the accumulation of the MVA pathway metabolites, IPP and DMAPP. To overcome the toxicity associated with exogenous expression of MVA pathway genes in *E. coli* BL21(DE3) cells, we employed a tight regulation strategy using CRISPRi; in seed

cultivations of lycopene-producing cells, the transcription of all the MVA pathway genes was tightly repressed by blocking the *lac* promoter (sgRNA(K1)) and utilization of acetyl-CoA, which is a starting metabolite for production of MVA production, was impeded by targeting *mvaE* gene using CRISPRi with the sgRNA(E2). This dual CRISPRi strategy significantly reduces the toxic effects that are otherwise present due to leaky expression of multiple exogenous genes and MVA pathway intermediates. Upon initiation of the main flask culture, L-rhamnose was diluted out in the growth media, thus relieving the repression and allowing induction of the heterologous MVA pathway.

To systematically investigate whether tight regulation of the MVA pathway could be used to uncouple cell growth from product formation, we first examined cell growth and GFP protein expression patterns following addition of various concentrations of L-rhamnose throughout the culture period. In seed cultures, 1 mM L-rhamnose was added to tightly repress GFP expression; L-rhamnose concentrations were then varied from 0 to 1 mM while transferring to the main flask cultures. This protocol allowed us to determine the dynamic range of CRISPRi-mediated repression. Evaluation of the repression function also revealed the minimum (16 μM L-rhamnose) and maximum repression levels (1 mM L-rhamnose) that can be obtained as a function of L-rhamnose concentration (Fig. 4A). There was no growth inhibition due to repression of the target genes or high concentration of L-rhamnose under any of the conditions (Fig. 4B). By 12 h of culture, we observed that higher concentrations of L-rhamnose resulted in stronger inhibition of GFP expression. After a delay of 180 min (OD_{600} = 0.6), GFP fluorescence changed depending on the L-rhamnose concentration, indicating that GFP expression was induced in exponentially growing *E. coli* cells (Fig. 4A). Based on these results, we cultivated BL21(DE3) cells capable of producing lycopene or (-)-α-bisabolol in seed cultures supplemented with 1 mM L-rhamnose (under this condition, the *lac* promoter and *mvaE* gene are repressed). The cells were then transferred into 0 mM L-rhamnose or kept in 1 mM L-rhamnose in the main culture flasks (Fig. 4C, left panel). When the seed cultures with 1 mM L-rhamnose were transferred to the main flask cultures without L-rhamnose, lycopene production increased 8.0-fold (71.4 ± 8.5 mg/L) compared to that in the control cells cultivated in the absence of L-rhamnose (8.9 ± 1.8 mg/L). Maintaining the cells in 1 mM L-rhamnose in both the seed and main flask cultures increased the lycopene production 5.7-fold (50.6 mg/L ± 10.1 mg/L). The growth of cells in all of these cases was virtually identical (Fig. 4C, right panel).

To test the general applicability of our tight regulation approach, we next focused our attention on another terpenoid, (-)-α-bisabolol, which is synthesized via the MVA pathway. (-)-α-Bisabolol is also known as levomenol and is an unsaturated sesquiterpene (C15) alcohol that is mainly manufactured by steam-distillation of the candeia essential oil extracted from the Brazilian candeia tree. It has been used in many cosmetic and pharmaceutical products because of its skin-soothing and anti-inflammatory properties. In order to produce (-)-α-bisabolol in *E. coli*, we replaced the lycopene synthase operon (*crtEIB*) of the pTM-CRT plasmid with a recently identified (-)-α-bisabolol synthase (BBS) enzyme isolated from German camomile (*Matricaria chamomilla*) (Son et al., 2014) (Fig. 2A). As with the lycopene production

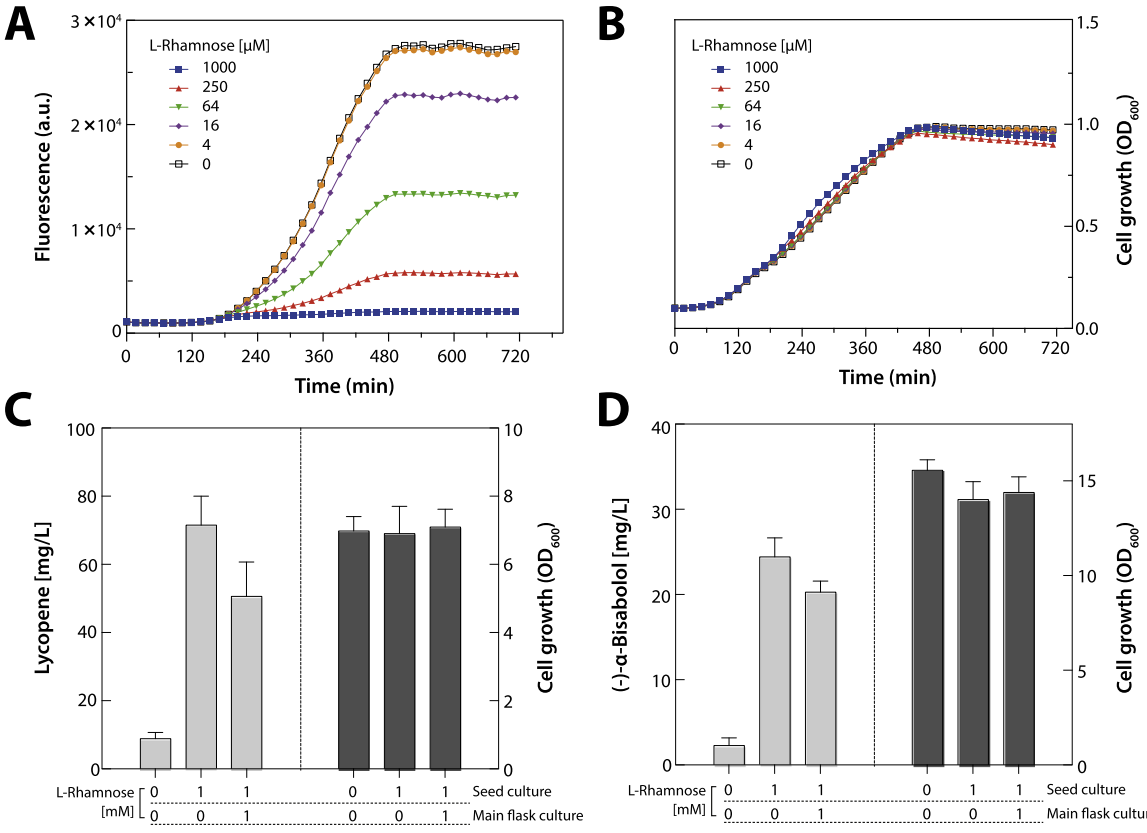

Fig. 4. Tight regulation strategy for enhanced production of terpenoids using CRISPRi. (A) Kinetics of L-rhamnose-dependent repression of target genes using the fluorescence reporter pREGFP3. (B) Effects of different concentrations of L-rhamnose on the growth of *E. coli* cells harboring the pREGFP3. (C, D) Tight regulation of *mvaE* gene expression using CRISPRi for enhanced lycopene and (-)-α-bisabolol production in *E. coli*, respectively. At the pre-culture stage, 1 mM L-rhamnose was added into LB media to induce dCas9 protein expression, which resulted in the nearly complete silencing of the expression of MVA pathway genes. At the production stage, L-rhamnose was diluted out when the cells in the seed culture were added to the medium for the main flask culture.

procedure, we tightly repressed MVA pathway genes in seed cultures by blocking both the *lac* promoter and *mvaE* gene using CRISPRi in the presence of 1 mM L-rhamnose. Similar to the case of lycopene production, (-)-α-bisabolol production in the main flask culture increased by up to 10.6-fold in the absence of L-rhamnose and by up to 8.8-fold in the presence of 1 mM L-rhamnose; the final biomasses (OD_{600}) of all the cultures was almost same under these conditions (Fig. 4D).

3.5. Coordination of cell growth and IPP/DMAPP accumulation

IPP and DMAPP are important metabolic intermediates involved in the production of different terpenoid end products. Addition of IPP units to a DMAPP core molecule, which is catalyzed by farnesyl diphosphate synthase (IspA), an enzyme that is essential to *E. coli* cell growth under aerobic conditions (Baba et al., 2006), results in the production of prenyl diphosphates of increasing length, including FPP (C15) and GGPP (C20). These molecules play key roles in biological processes required for cell survival, including cell wall biosynthesis (bactoprenol), membrane function (hopanoids, ether-type membrane lipids), and electron transport (heme, ubiquinone, menaquinone) (Boronat and Rodríguez-Concepción, 2015). Reducing the IPP and DMAPP pool available for these pathways would therefore inhibit cell growth. To examine whether fine-tuning the expression level of the *ispA* gene using the CRISPRi system could balance the metabolic flux between cell growth and IPP/DMAPP accumulation, we generated sgRNAs repressing the endogenous *ispA* gene in isoprene-producing *E. coli* BL21(DE3) cells harboring the biosynthetic MVA pathway genes and an isoprene synthase gene from the poplar tree (*Populus trichocarpa*) (Sasaki et al., 2005) (Fig. 2A). Isoprene is an important starting material for the synthesis of rubber, elastomers, and medicines (Xue and Ahring, 2011). There is a competition between isoprene production and prenyl diphosphate production from DMAPP (Silver and Fall, 1991). We confirmed high repressor efficacy toward *ispA* in our gene reporter assay and by quantifying mRNA expression (Fig. 5A and B). Repression of *ispA* expression with 1 mM L-rhamnose increased isoprene production by 2.6-fold (Fig. 5C, left) and CRISPRi-guided *ispA* repression further enhanced isoprene production when MVA pathway cassettes in which the *mvaE* gene contained an engineered RBS were used, although the isoprene titer was lower than that obtained with BL21(DE3) harboring wild-type MVA pathway genes (Fig. 5C, right). This suggests that, regardless of high (WT RBS of *mvaE*) or low flux (RBSD variant of *mvaE*) for MVA synthesis, *ispA* repression plays a key role in improving IPP/DMAPP accumulation and subsequent isoprene production. Because *ispA* is an essential gene in *E. coli*, we further investigated the effects of *ispA* repression on cell growth by monitoring OD_{600} for 11 h using a microplate reader. In the absence of exogenous isoprene production, 1 mM L-rhamnose-induced repression of *ispA* expression in *E. coli* BL21(DE3) cells reduced the cell growth rate by 80%, whereas expression of the

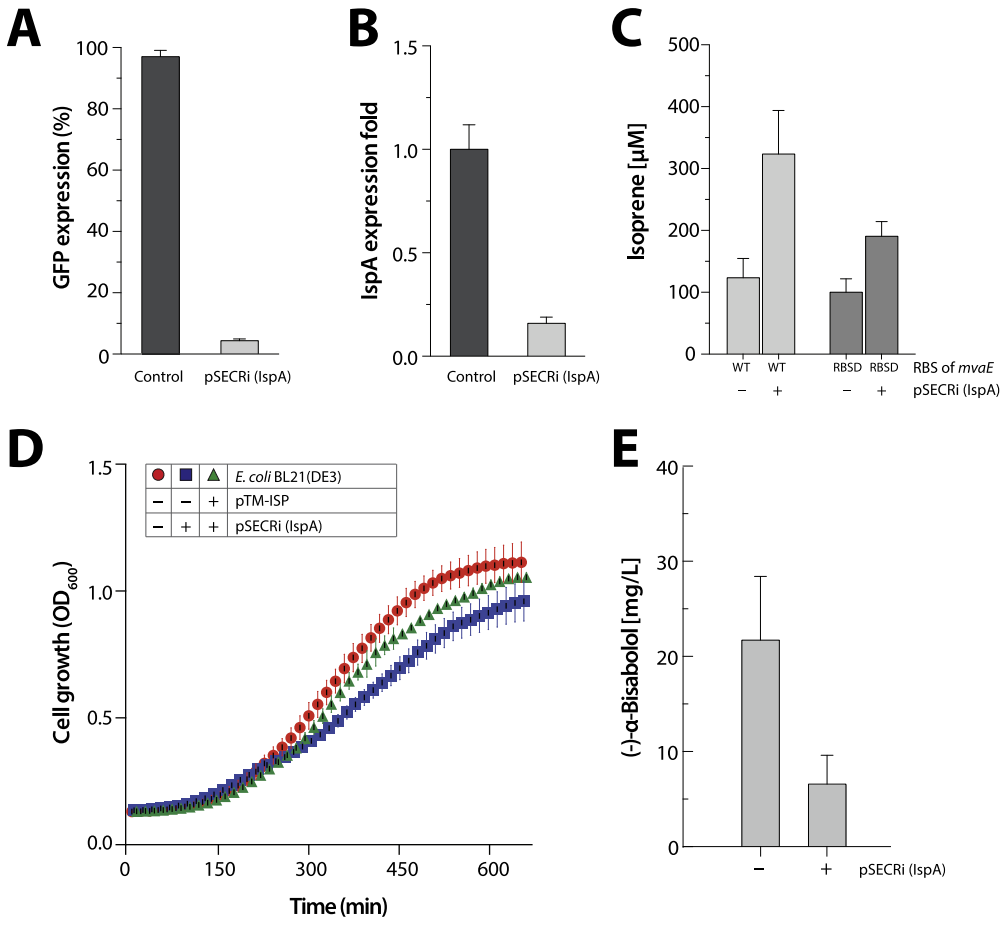

Fig. 5. Coordination of the regulation of essential genes with terpenoid production. (A) Control of essential gene (*ispA*) expression using pSECRi (IspA). (B) Validation of *ispA* mRNA expression level by qRT-PCR. (C) Effects of *ispA* repression on isoprene production from *E. coli* cells harboring wild-type MVA pathway and an RBS-engineered MVA pathway. (D) Growth of *E. coli* cells producing isoprene with or without *ispA* repression. *E. coli* BL21(DE3) cells harboring the pTM-ISP and pSECRi (IspA) plasmids (▲); BL21 (DE3) containing the pTrc99A and pSECRi (IspA) plasmids (■); BL21(DE3) with the pTrc99A and pSEVA221 plasmids (●The circle symbol is too smaller than other symbols, triangle and square.). (E) (-)-α-Bisabolol production following CRISPRi-mediated *ispA* repression using the pSECRi (IspA).

MVA pathway and *ispS* genes restored the growth rate to 95% during *ispA* repression (Fig. 5D). This indicated that essential metabolites (IPP and DMAPP) are balanced for cell growth and isoprene production. In contrast to isoprene production, *ispA* repression decreased terpenoid production because a precursor for terpenoid synthesis, FPP, is synthesized by sequential condensation reactions of DMAPP and two IPPs catalyzed by IspA. Further, as expected, *ispA* repression decreased (-)-α-bisabolol production by 3.3-fold following 48 h of cultivation (Fig. 5E).

4. Discussion

Nature has evolved highly efficient ways for balancing gene expression in living cells that utilize highly dynamic and complex metabolic systems. The elaborate harmony of protein expression levels is key for correct working of biological systems (Scott et al., 2010) and is often essential for the efficiency of metabolic pathways (Na et al., 2010). However, in contrast to native biological systems, the expression of a synthetic system can lead to imbalances in gene expression and subsequent formation of by-products; accumulation of these by-products, some of which are toxic, can inhibit host cell growth (Koffas et al., 2003). Therefore, the expression level of enzymes in biosynthetic metabolic pathways should be tuned for optimal function (Son et al., 2014). In particular, balancing the expression level of multiple genes in metabolic engineering is crucial for increasing the productivity of biosynthetic pathways and subsequently for sustainable production of valuable products.

Here, we have demonstrated that the regulatable CRISPRi system could be used in the context of metabolic engineering for terpenoid production to regulate the expression of multiple exogenous genes and endogenous essential genes in a dynamic and quantitative manner. To this end, we established a single CRISPRi plasmid harboring a derivative of RNA-guided DNA endonuclease, dCas9, which lacks nuclease activity, and an sgRNA targeting the gene of interest (Fig. 1A). While conventional tools for gene knockout are laborious and costly, the knockdown of genes using the regulatable CRISPRi system only needs co-expression of the CRISPRi plasmid, thus making it very simple and efficient (Qi et al., 2013). Our CRISPRi plasmid expresses dCas9 protein and sgRNA in a single plasmid. We designed this plasmid is based on the low copy number pSEVA series in order to minimize metabolic burden; moreover, this plasmid can be easily swapped with other antibiotics and replication origins for use in applications with other bacterial hosts (Silva-Rocha et al., 2013). However, it is important to note that the functionality of the L-rhamnose promoter and J23119 promoter must be determined on a case-by-case basis in other bacteria. To tightly control dCas9 expression, we used an L-rhamnose-inducible promoter with RhaS and RhaR regulators but 1.8 kb of RhaS and RhaR regulators in CRISPRi plasmids can be removed to reduce plasmid size without regulatory functionality (Wegerer et al., 2008). IPTG-inducible promoters have been widely used for the expression of heterologous genes in metabolic engineering efforts. We have also used the IPTG-inducible promoters (P_{lac} and P_{trc}) to control the expression of terpenoid synthases and synthetic MVA pathways (Fig. 2A). Therefore, we chose the L-rhamnose-inducible and J23119 promoter for an orthogonal control of the transcription of *S. pyogenes* dCas9 gene and sgRNA, respectively, in the presented CRISPRi system. Moreover, the L-rhamnose-inducible promoter is capable of homogenous and rheostatic control of transcription of heterologous genes and shows undetectable background expression in the absence of L-rhamnose (Giacalone et al., 2006; Wegerer et al., 2008).

Further, to exchange the target sequence, only a single cloning step using simple PCR and ligation was required. For multiplex silencing using CRISPRi, we constructed sgRNA arrays using a modular assembly method that was previously described for generating artificial zinc finger arrays (Wright et al., 2006). Thus, we placed sgRNAs in tandem, with *Age*I and *Xma*I restriction enzyme sites, inserted at both ends of the sgRNA cassette, or by using the Gibson assembly method (Fig. S6). *Age*I and *Xma*I have compatible cohesive ends, and the ligation of both DNA fragments digested with *Age*I and *Xma*I generates a new restriction site that is not cleavable by either of the two restriction enzymes.

For CRISPRi-guided balancing of metabolic pathways, we decided to use an L-rhamnose-inducible promoter to achieve controlled expression of dCas9. This allowed dose-dependent repression of transcription and was not associated with overt toxicity. We were particularly keen to evaluate the utility of the CRISPRi system to reduce the expression of an endogenous essential gene, *ispA* (Baba et al., 2006), since the reversible nature of this system would permit survival of cells; this is in contrast to conventional recombination-based tools that permanently delete genes. We observed that selective knockdown of IspA activity using the CRISPRi system mitigated carbon loss to GPP and FPP during isoprene production in engineered *E. coli* cells and that cell growth was not inhibited under these conditions (Fig. 5). These important results suggested that variations of CRISPRi that incorporate inducible or dynamically controlled arrays would create new paradigms for metabolic engineering, as they will allow the dynamic control of the expression of target (essential) genes without having a negative impact on growth.

The CRISPRi system can be easily adjusted for different target gene sequences by simply changing the sgRNA sequence in a single repression plasmid, which requires replacing only a 20-bp sequence. This makes the CRISPRi system very convenient to engineer, particularly if generation of plasmids that target multiple genes is required. Taking advantage of this feature of the CRISPRi system, we screened for key metabolic control points of target metabolic pathways, which allowed us to define how the abundance of an enzyme or transcript relates to improved production of a metabolite of interest. As a proof-of-concept, we focused on terpenoid production pathways. Although *E. coli* encodes a native MEP pathway, additional expression of MEP pathway genes or genes in the MVA pathway is required for enhanced production of various terpenoids in *E. coli* (Martin et al., 2003; Yoon et al., 2006; Zhao et al., 2011). Furthermore balanced expression of MVA pathway enzymes is required, since HMG-CoA and IPP are both toxic in *E. coli* (Kizer et al., 2008; Martin et al., 2003). We designed sgRNAs to inhibit the transcription of all six MVA pathway enzymes (K1–K3), an MVA input flux enzyme, MvaE, for MVA pathway flux inhibition (E1–E4), or MvaS enzyme that converts acetoacetyl-CoA to HMG-CoA (S1 and S2). We found that all the sgRNAs successfully regulated nine sites in genes of the biosynthetic MVA pathway, and even when the target sequences that were ~4 kb away from the translational start site (Fig. 2). Single repression of individual genes (*mvaK1*, *mvaE*, and *mvaS*) revealed that the *mvaE* gene is the primary determinant of the flux to the IPP and DMAPP metabolites, which are the universal precursors of all terpenoid compounds. Furthermore, blockage of the upstream region of MVA pathway clusters using sgRNA(K1) affected the expression of all the downstream genes, thus demonstrating the feasibility of controlling MVA pathway flux through silencing a promoter in an operon context. Targeting a promoter could silence many or all of the enzymes in a biosynthetic pathway because genes encoding the related enzymes, which are often gathered into operons, are transcribed as polycistronic mRNA.

To create a metric to assess the repression efficiency of the CRISPRi system, we performed two fluorescent-based assays that report the activity of single genes or operons. Such reporters offer a simple workflow to examine the activity of individual sgRNA

activity in terms of protein abundance, a metric that should be more meaningful for immediate practical applications and scientific discovery. Interestingly, we found that the sgRNAs (K1, K2, and K3) repressing all the MVA pathway enzymes showed different repression efficiencies in two operon reporter assays (Fig. 2D and E); sgRNAs controlling the *mvaK1-mvaD-mvaK2-idi-mvaE-gfp* operon showed less efficient repression than that observed in the same sgRNAs repressing the *mvaK1-gfp* operon (Table S4), suggesting that unintended internal promoter activity exists within the *mvaK1-mvaD-mvaK2-idi-mvaE* operon of the biosynthetic MVA pathway. Previously it was reported that unknown promoter activity of the biosynthetic MVA pathway regulates downstream enzymes (Kawano et al., 2005; Redding-Johanson et al., 2011). To verify this notion, we conducted two kinds of operon reporter assay using additionally constructed fluorescence reporter plasmids (Fig. S7). After removing the *lac* promoter or both the *lac* promoter and the *mvaK1* gene using the pREGFP4 reporter plasmid, the fluorescence almost disappeared (Fig. S7A), which indicated that GFP expression is under the control of the *lac* promoter. However, 26% of the fluorescence remained after removing the *lac* promoter from the pREGFP5 whereas only 2.5% of the original fluorescence was observed when both the *lac* promoter and all the MVA genes (*mvaK1-mvaD-mvaK2-idi-mvaE*) were removed (Fig. S7B). This implied that the *mvaD-mvaK2-idi-mvaE* operon has an unintended promoter activity. Indeed, in silico analysis suggested that the *mvaK2* gene of *Streptomyces pneumoniae* used in this study contains highly conserved TTGTCA (-30 box) and TATAAT (-10 box) sequences. Therefore, we used both sgRNA(K1) and sgRNA(E2)s in order to fine-tune the balance of MVA pathway enzymes expressed in our system.

One advantage that the CRISPRi system has over knockout methods is that CRISPRi-based knockdown is reversible in many cases (Qi et al., 2013). Taking advantage of this effect, we established a tight regulation strategy to enhance the production of target terpenoids. We encoded the dCas9 gene under the control of the L-rhamnose-inducible promoter, and the target-specific sgRNA was constitutively expressed in *E. coli* cells. This allowed us to minimize leaky expression of MVA pathway genes by adding L-rhamnose, which otherwise generates toxic intermediates and hinders bacterial growth (Kim et al., 2009). Removal of L-rhamnose during the main flask culture restored gene expression and increased the production of lycopene and (-)-α-bisabolol (Fig. 4). CRISPRi-assisted biosynthesis of terpenoids was thus implemented on a biosynthetic metabolic pathway and did not require any specialized knowledge of the structure or function of any enzyme in multigene pathways or modification of the genetic structure of biosynthetic production pathways. Further, the implementation of this tight regulation approach was simple and is applicable to nearly any biosynthetic pathway, especially those that generate toxic intermediates.

In summary, the CRISPRi system holds great promise as a general genetic programming platform that is suitable for microbial metabolic engineering. It holds advantages over traditional gene-knockout strategies because of its ability to reversibly repress genes essential for growth. Furthermore, because of its high specificity with regard to the regulation of single or multiple genes, this RNA-guided system is more productive than antisense RNA strategies. Moreover, the ability of the CRISPRi system to uncouple enhanced production of valuable metabolites from negative impact on cell growth renders it suitable for the efficient production of many other terpenoids.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ymben.2016.08.006.

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