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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.

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,