Metabolic Engineering 40 (2017) 115–123

Contents lists available at ScienceDirect

Metabolic Engineering

journal homepage: www.elsevier.com/locate/ymben

Improving key enzyme activity in phenylpropanoid pathway with a designed biosensor

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ARTICLE INFO

Keywords:
Biosensor
p-Coumarate:coenzyme A ligase
High-throughput screening
Phenylpropanoids pathway
Protein engineering
Resveratrol

ABSTRACT

Overexpressing key enzymes of biosynthetic pathways for overproduction of value-added products usually imposes metabolic burdens on cells, which can be circumvented by improving the key enzyme activities. *p*-Coumarate: CoA ligase (4CL) is a critical enzyme in the phenylpropanoid pathway that synthesizes various natural products. To screen for 4CL with improved activity, a biosensor of resveratrol whose biosynthetic pathway involves 4CL was designed by engineering the TtgR regulatory protein. The biosensor exhibited good specificity and robustness, allowing rapid and sensitive selection of resveratrol hyper-producers. A 4CL variant with improved activity was selected from a 4CL mutagenesis library constructed in the resveratrol biosynthetic pathway in *Escherichia coli*. This mutant led to increased production of not only resveratrol but also the flavonoid naringenin, when introduced in their corresponding biosynthetic pathways. These findings demonstrate the feasibility of improving key enzyme activities in important biosynthetic pathways with the aid of designed biosensors of pathway products.

1. Introduction

Heterogeneous production of value-added compounds, using hosts such as *Escherichia coli* and yeast, provides a rapid and robust access to the desired products (Keasling and Chou, 2008; Kirby and Keasling, 2009). Based on the heterogeneous reconstitution of the biosynthetic pathway, process optimization is essential to maximize production. Metabolic and protein engineering strategies are efficient approaches for developing hyper-producing variants by circumventing potential barriers to direct the carbon flow towards the desired products, such as suboptimal performance of key enzymes in the pathways (Chen et al., 2015; Martin et al., 2003). As protein overexpression is a major metabolic burden on engineered cells (Wu et al., 2016), designing enzymes with higher specific activity is the most effective way to improve pathway efficiency without undermining cellular functions.

p-Coumarate:CoA ligase (4CL) is a critical enzyme in the phenylpropanoid pathway that catalyzes the synthesis of CoA thioesters of *p*-coumarate, caffeic acid, ferulic acid and sinapic acid (Ehlting et al., 1999), which are precursors for a variety of plant secondary metabolites (Fig. S1). Improving the activity of 4CL has important implications. Since pathway enzyme performance is always limited by intracellular availability of substrates and cofactors, and excessive accumulation of biosynthetic intermediates can be toxic to the cells, *in vivo* directed evolution is a more advantageous strategy for metabolic pathway enzyme engineering than rational design, since mutant enzymes overcoming a specific *in vivo* limitation can be selected. High-throughput screening techniques for the biosynthesized product can greatly improve the efficiency of the engineering process.

Transcription factor-based *in vivo* small-molecule biosensors have found applications in high-throughput screening for specific products (Raman et al., 2014; Siedler et al., 2014). However, the lack of available transcription factors that respond to small molecules of interest has limited their applications. To this end, the effector specificities of regulatory proteins have been altered in some cases (Galvao and de Lorenzo, 2006; Tang et al., 2008; Taylor et al., 2016), however, only a few have been confirmed as functional *in vivo* biosensors in practice (Chen et al., 2015; Tang and Cirino, 2011; Tang et al., 2013). Specificity, sensitivity and robustness are important features for high-

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http://dx.doi.org/10.1016/j.ymben.2017.01.006
Received 24 October 2016; Received in revised form 9 December 2016; Accepted 18 January 2017
Available online 19 January 2017

quality *in vivo* biosensors.

In this study, the TtgR regulatory system from *Pseudomonas putida* (Espinosa-Urgel et al., 2015; Teran et al., 2003) was adapted into *E. coli* and the TtgR protein was engineered to respond to resveratrol. As 4CL is involved in the biosynthesis of resveratrol (Lim et al., 2011), this TtgR variant served as an efficient *in vivo* resveratrol biosensor used to identify 4CL variants with enhanced activity in *in vivo* directed evolution. The improved 4CL variant will greatly benefit the overproduction of a variety of valuable compounds (Fig. S1). Our work has explored the approaches of designing high-quality *in vivo* biosensors for small molecules and applying them in improving the activities of key enzymes in important biosynthetic pathways.

2. Materials and methods

2.1. General

Restriction enzymes and DNA polymerases were purchased from Takara Bio Inc. (Dalian, China). T4 DNA ligase and *Dpn*I were purchased from New England Biolabs (Beijing, China). Oligonucleotides were synthesized by Life Technologies (Shanghai, China). *p*-Coumaric acid, naringenin, genistein and resveratrol, malonyl-CoA, CoA and adenosine-5′-triphosphate (ATP) were all purchased from Sigma-Aldrich (St. Louis, USA).

E. coli strains BL21(DE3) and BW25113 were used for protein overexpression and resveratrol production, respectively. Resveratrol and naringenin production was performed in yeast extract M9 (YM9) medium as described previously (Lim et al., 2011), while other cultures were done in Luria-Bertani (LB) medium if not specially mentioned. The antibiotics ampicillin (100 μg/mL) and kanamycin (50 μg/mL) were used when necessary.

2.2. Plasmid construction

Sequences for all primers are listed in Supplementary Table S1. All plasmids used in this study are shown in Table S2.

1) **pTtg.** Using plasmid pHY (Liang et al., 2015) as template, amplification was performed with primers pTtgR-(−35)-*Sac*II-for and pTtgR-(−35)-*Sac*II-rev. The PCR product was digested with *Sac*II and then self-ligated, resulting in plasmid pTtg. The mutations in the promoter region were introduced with Stratagene QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies Inc, Santa Clara, USA).
2) **pTtgR.** The *ttgR* gene (Genbank accession NO.AF238479.1) was amplified using primers TtgR-*Nde*I-for and TtgR-*Sac*I-rev, with the genomic DNA of *Pseudomonas putida* DOT-T1E as template. After digested with *Nde*I and *Sac*I, the *ttgR* gene was ligated into the PCR product amplified with primers pTtg-linearized-*Nde*I-for and pTtg-linearized-*Sac*I-rev using plasmid pTtg as template, resulting in plasmid pTtgR.
3) **pET28a-4CL.**The gene *At4CL1* encoding *p*-coumarate:CoA ligase (4CL)(Genbank accession NO. U18675.1) was amplified from *Arabidopsis thaliana* cDNA using primers At4CL-*Nde*I-for and At4CL-*Xho*I-rev, and the PCR product was ligated to vector pET28a after digestion with *Nde*I and *Xho*I, resulting in plasmid pET28a-4CL.
4) **pET28a-STS**. The *STS* gene from *Vitis vinifera* (Genbank accession NO. EF192465.1) was synthesized by GENEWIZ (Suzhou, China) after codon optimization (Table S3). Primers VvSTS-*Nco*I-for and VvSTS-*Xho*I-rev were used to amplify the *STS* gene, and the PCR product was then ligated to vector pET28a after digestion with *Nco*I and *Xho*I, resulting in plasmid pET28a-STS.
5) **pGAP-4CL-STS and pGAP-STS-4CL.** The constitutive promoter GAP (promoter of the *gapA* gene encoding glyceraldehyde-3-phosphate dehydrogenase in *E. coli*)(Lim et al., 2011) was amplified from the genomic DNA of strain *E. coli* MG1655 with primers P_{GAP}-*Kpn*I-for and P_{GAP}-*Nde*I-rev, and the PCR fragment was digested with *Kpn*I and *Nde*I, and then ligated to vector pSB3K5 (Shetty et al., 2008), resulting in plasmid pGAP.

   The *At4CL1* gene fragment amplified with primers At4CL-*Nde*I-for and At4CL-rbs-*Hind*III-rev was overlaped with the *STS* gene fragment amplified with primers VvSTS-rbs-*Hind*III-for and VvSTS-*Sac*I-rev, and the overlaped PCR fragment was digested with *Nde*I and *Sac*I and ligated to plasmid pGAP, resulting in plasmid pGAP-4CL-STS.

   The *STS* gene fragment amplified with primers VvSTS-*Nde*I-for and VvSTS-rbs-*Hind*III-rev was overlaped with the *At4CL1* gene fragment amplified with primers At4CL-rbs-*Hind*III-for and At4CL-*Sac*I-rev, and the overlaped PCR fragment was digested with *Nde*I and *Sac*I and ligated to plasmid pGAP, resulting in plasmid pGAP-STS-4CL.
6) **pGAP-STS and pGAP-4CL.** PCR was performed using plasmid pGAP-STS-4CL as template with primers VvSTS-*Nde*I-for and VvSTS(489TAA)-rev, the PCR product was used as the megaprimers to perform the MEGAWHOP PCR (Miyazaki, 2011) using plasmid pGAP-STS-4CL as template. After the MEGAWHOP PCR, *Dpn*I (20 U) digestion was performed at 37 °C for 2 h, then *Dpn*I was inactivated at 80 °C for 20 min. Then the PCR products were used to transform strain *E. coli* MC1061, resulting in plasmid pGAP-4CL. PCR was performed using plasmid pGAP-STS-4CL as template with primers At4CL-rbs-*Hind*III-for and At4CL(958TAA)-rev, the PCR product was used as the megaprimers to perform the MEGAWHOP PCR using plasmid pGAP-STS-4CL as template described above, resulting in plasmid pGAP-STS.
7) **pGAP-CHS-4CL.** The *CHS* gene encoding chalcone synthase from *Petunia x hybrid* (Genbank accession NO. KF765781.1) was synthesized by GENEWIZ. Primers PhCHS-*Nde*I-for and PhCHS-*Hind*III-rev were used to amplify the *CHS* gene, and the PCR product was digested with *Nde*I and *Hind*III and ligated to plasmid pGAP-STS-4CL carrying the genes encoding wild-type 4CL1 or its 4AT mutant, resulting in plasmid pGAP-CHS-4CL carrying the genes encoding wild-type 4CL1 or its 4AT mutant, respectively.

2.3. Strain construction

All strains used in this study are shown in Table S2. The *lacZ* gene was amplified from the genomic DNA of strain MG1655 with primers *lacZ-Kpn*I-for and *lacZ-Bgl*II-rev and ligated into plasmid pTtgR carrying gene encoding the TtgR mutant mu3, after digestion with *Kpn*I and *Bgl*II. Then the fragment containing $P_{ttgABC2}$-*lacZ* and gene encoding mu3 mutant was amplified with primers pAH156-$P_{ttgABC2}$-for and pAH156-*ttgR*-rev, and then assembled with the PCR fragment amplified with primers *ttgR*-pAH156-for and $P_{ttgABC2}$-pAH156-rev using the CRIM plasmid pAH156 (Haldimann and Wanner, 2001) as template, with Gibson Assembly (Gibson et al., 2009). The construct was then integrated into the *E. coli* BW25113 chromosome using helper plasmid pAH69 (Haldimann and Wanner, 2001), resulting in strain M3. The integration was verified by PCR.

2.4. Library construction

The random mutagenesis libraries of TtgR and 4CL1 were all constructed through error-prone PCR. To construct the TtgR library, the primers TtgR-*Nde*I-for and TtgR-*Sac*I-rev were used to amplify the *ttgR* gene, using plasmid pTtgR as template. The PCR reaction mixture consisted of 7 mM $MgCl_2$, 0.2 mM each of dATP and dGTP, 1 mM each of dCTP and dTTP, 0.025 mM $MnCl_2$ and rTaq DNA polymerase. The PCR product was used as the megaprimers to perform the MEGAWHOP PCR using pTtgR as template. After the MEGAWHOP PCR, *Dpn*I (20 U) digestion was performed at 37 °C for 2 h, then *Dpn*I was inactivated at 80 °C for 20 min. Then the PCR products were used