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Improving key enzyme activity in phenylpropanoid pathway with a designed biosensor

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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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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

to transform strain *E.coli* MC1061 and around 10^6 transformants were recovered. Ten randomly picked clones were sequenced and contained an average of 1.5 nucleotide mutations per clone. The random mutagenesis library of 4CL1 was constructed as described above using plasmid pGAP-STS-4CL as the template, with primers At4CL-rbs-*Hind*III-for and At4CL-*Sac*I-rev. Sequencing results revealed 3 nucleotide mutations per clone. For each library, all colonies from the agar plates were used for plasmid isolation to prepare the plasmid library.

2.5. TtgR library screening

The TtgR plasmid library was used to transform strain BW25113 (10^7 transformants were recovered). Fluorescence-activated cell sorting (FACS) was performed on a FACSAria II sorter (BD, San Jose, USA). Fluorescence was excited at 561 nm and the fluorescence emission was detected using a 610/20 nm band-pass filter. In the first round of negative screening, cells were precultured overnight in LB medium and then diluted to OD_{600}=0.2 in the same medium in the absence of inducer. The cells were then grown for 12 h and the least fluorescent 8.0×10^5 cells were collected from a total of 1×10^6 cells (representing 80% of all cells sorted). This was performed to eliminate TtgR variants with high levels of leaky expression. The collected cells showing low fluorescence were subjected to another negative screen in the presence of 0.1 mM *p*-coumaric acid to eliminate clones that were induced by *p*-coumaric acid. The collected cells were then induced with 0.1 mM resveratrol and grown for 12 h to enable positive screening, and the most fluorescent 1.0×10^4 cells were sorted from a total of 1×10^6 cells. This procedure was repeated in a second round of negative and positive screens. This dual screening was repeated four times, followed by a final round of negative screening in the absence of inducer. Twenty clones were selected for rescreening in test tubes in the presence of 0 mM, 0.1 mM and 0.3 mM resveratrol.

2.6. 4CL1 library screening

The random mutagenesis library of 4CL1 was used to transform strain *E. coli* M3. After incubated at 37 °C for 19 h on YM9 agar containing 985 mg/L *p*-coumaric acid, 20 μg/mL X-GAL and 50 μg/mL kanamycin, the darkest blue colonies (by the eye) were selected for quantifying resveratrol production in liquid culture.

2.7. RFP fluorescence assays

A single colony of strain BW25113 harboring plasmid pTtgR was grown overnight in LB medium at 37 °C and then diluted to OD_{600}=0.02 in the same medium containing an appropriate concentration of resveratrol, and allowed to grow under inducing condition at 37 °C for 12 h. The OD_{600} and RFP fluorescence emission were measured with a SynergyMx Multi-Mode Microplate Reader (BioTek, Vermont, USA) (556 nm excitation filter and 586/20 nm emission filter).

A single colony of strain BW25113 harboring plasmids pTtgR and pGAP-STS-4CL was grown overnight in LB medium at 37 °C and then diluted to OD_{600}=0.02 in YM9 medium containing an appropriate concentration of *p*-coumaric acid, and allowed to grow at 37 °C for 12 h. The OD_{600} and RFP fluorescence emission were measured as described above.

2.8. HPLC analysis

E. coli strain M3 expressing the biosynthetic pathway of resveratrol or naringenin was grown overnight in LB medium at 37 °C, and then diluted to OD_{600}=0.02 in YM9 medium containing *p*-coumaric acid. For resveratrol production, the strains were cultured in the presence of 985 mg/L *p*-coumaric acid at 37 °C for 24 h. While for naringenin production, the strains were cultured in the presence of 492 mg/L *p*-coumaric acid at 30 °C for 60 h. 500 μL sample was harvested and the same volume of ethanol was added and mixed (Koopman et al., 2012). The sample was filtered with a 0.2 μm membrane filter and then analyzed by HPLC using Shimadzu LC-20A system equipped with a photodiode array detector (Shimadzu Corp., Kyoto, Japan). A Waters Symmetry C18 column (250×4.6 mm, 5 μm) working at 35 °C was used for all analyses. Separation of *p*-coumaric acid, bisnoryangonin and resveratrol was achieved using a mobile phase of 35% acetonitrile (containing 0.1% formic acid) at a flow rate of 0.5 mL/min. *p*-Coumaric acid and resveratrol were monitored at 305 nm, and bisnoryangonin was monitored at 360 nm. For separation of naringenin, a mobile phase of 50% acetonitrile (containing 0.1% formic acid) at a flow rate of 0.5 mL/min was used, and naringenin was monitored at 288 nm. The productions were calculated with calibration curves prepared with the commercially available authentic resveratrol and naringenin.

The resveratrol, bisnoryangonin or naringenin peak was collected on semi-preparative HPLC (LC3000 HPLC system, Beijing Chuang Xin Tong Heng Science & Technology Co., Ltd, Beijing, China) and verified on Agilent Accurate-Mass-Q-TOF MS 6520 system equipped with an electrospray ionization source (Agilent Technologies, Santa Clara, USA) in the positive ionization mode.

2.9. Protein purification

A single colony of strain BL21(DE3) harboring plasmid pET28a-4CL carrying of wild-type or mutant 4CL1 was grown in LB medium at 37 °C and induced with 0.4 mM IPTG when OD_{600} reached 0.6, then the culture was continuously grown at 30 °C for 12 h. Cells were harvested by centrifugation at 4 °C and resuspended in the lysis buffer (50 mM Tris–HCl, 300 mM NaCl, 10 mM imidazole, pH 8.0) and disrupted by sonication with a JY92-IIN Ultra Sonic Cell Crusher (Ningbo, China). Cell debris were removed by centrifugation (9000*g*, 10 min, 4 °C) and the supernatants were loaded on a pre-equilibrated nickel-nitrilotriacetic acid (Ni-NTA) column (Qiagen, Valencia, USA). The column was washed with the wash buffer (50 mM Tris–HCl, 300 mM NaCl, 20 mM imidazole, pH 8.0), and the bound protein was then eluted with the elution buffer (50 mM Tris-HCl, 300 mM NaCl, 250 mM imidazole, pH 8.0). Imidazole was removed by dialysis at 4 °C against 100 mM Tris–HCl buffer (pH 8.0).

The STS enzyme was purified as described above and finally imidazole was removed by dialysis at 4 °C against 20 mM HEPES buffer (5 mM EDTA, pH 8.0).

The purity of proteins were assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and the protein concentrations were determined with Bradford method (Bradford, 1976).

2.10. 4CL1 activity assay and kinetic parameters determination

A 200 μL reaction mixture includes 5 μM *p*-coumaric acid, 5 mM ATP, 0.33 mM CoA,1 mM $MgCl_2$ and 2.0 μg purified 4CL1 protein in 100 mM Tris-HCl buffer (pH 8.0). Reaction was performed at 30 °C (Knobloch and Hahlbrock, 1977). The *p*-coumaroyl-CoA formation was monitored at 333 nm with a SynergyMx Multi-Mode Microplate Reader (BioTek, USA). One unit of activity was defined as the amount of enzyme that catalyzes the production of 1 μmol of *p*-coumaroyl-CoA per minute. The molar absorption coefficient of *p*-coumaroyl-CoA is 21,000 L mol^{-1} cm^{-1} (Stockigt and Zenk, 1975).

The enzyme kinetic parameters were determined by Lineweaver-Burk plots. The 4CL1 activity assay was performed as described above at 1–500 μM *p*-coumaric acid.

2.11. Bisnoryangonin production by purified 4CL1 and STS enzymes

The reaction mixture contains 500 μM *p*-coumaric acid, 20 mM ATP, 25 mM $MgCl_2$, 1 mM CoA and 0.5 mM malonyl CoA in 500 mM Tris-HCl buffer (pH 8.0). Purified wild-type 4CL1 (8.4 μg), STS (6 μg)

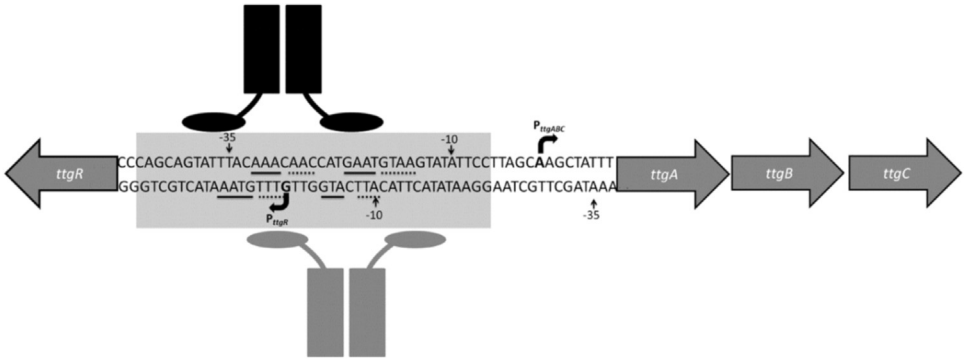

Fig. 1. Two TtgR protein dimers binding to pseudo-palindromic DNA sequences (dark or long dashed underline) in the absence of inducer. The 40-bp sequence in shaded font represents the TtgR binding site.

or both enzymes was added to the reaction mixture individually. The reactions were performed at 30 °C for 30 min.

3. Results

3.1. Adaptation of the TtgR regulatory system in E. coli

TtgR is a member of the TetR family of transcriptional repressors that regulates expression of the TtgABC efflux pump in *Pseudomonas putida* DOT-T1E, which is responsible for resistance to multiple antibiotics and plant secondary metabolites (Teran et al., 2003). In the absence of an inducer, the TtgR protein binds to the promoter of the *ttgABC* operon and inhibits its transcription (Fig. 1). The operon is derepressed when inducers (which are also substrates of the TtgABC efflux pump) bind to and release TtgR from the promoter. To reconstruct the TtgR regulatory system in *E. coli*, the 40-bp DNA sequence containing the promoter of the *ttgABC* operon ($P_{ttgABC1}$) was cloned upstream of red fluorescent protein (RFP), but it was not able to initiate the RFP expression in *E. coli* (Fig. S2a, Table S4). To make the promoter functional, after comparison with the promoter sequences of CP6 (Jensen and Hammer, 1998) and *lac* promoters frequently used in *E. coli*, T/A and A/G substitutions were independently introduced at the −10 position of $P_{ttgABC1}$, yielding promoters $P_{ttgABC2}$ and $P_{ttgABC3}$, respectively (Table S4). $P_{ttgABC2}$ carrying the T/A substitution was found to initiate RFP expression (Fig. S2b,c). The constitutively expressed TtgR was then integrated into plasmid pTtg harboring $P_{ttgABC2}$, yielding plasmid pTtgR in which RFP expression was repressed, indicating successful binding of TtgR protein to the promoter sequence (Fig. S2d). The reconstructed TtgR regulatory system containing $P_{ttgABC2}$-controlled RFP and constitutively expressed TtgR protein was introduced into the *E. coli* BW25113 strain. The induction effect of the natural inducer of TtgR, genistein, was confirmed, indicating that this regulatory system was functional in *E. coli* (Fig. S3).

3.2. Engineering TtgR for altered effector specificity

The crystal structure of TtgR revealed a monomeric protein with N-terminal DNA-binding and C-terminal ligand-binding domains (Alguel et al., 2007). To develop a TtgR variant that could bind resveratrol and activate expression from promoter $P_{ttgABC2}$, we constructed a random mutagenesis library of TtgR that was subjected to iterative fluorescence-activated cell sorting in combination with $P_{ttgABC2}$-RFP construct. From a total of 1.0×10^6 transformants in the library, we selected variants responding to resveratrol but not *p*-coumaric acid. Among them variant mu3 showed the highest induction fold towards resveratrol (Fig. S4). Although some mutants such as mutant mu5 and mu7 exhibited higher RFP induction levels in the presence of resveratrol, they also showed high leaky expressions in the absence of inducer, resulting in lower induction folds as compared with the mu3 mutant. For strain BW25113 expressing variant mu3, RFP fluorescence increased as a function of resveratrol concentration, while wild-type TtgR exhibited little response towards resveratrol (Fig. 2a). The half-maximal induction response occurred at approximately 0.23 ± 0.11 mM resveratrol. Flow cytometry analysis revealed that cells expressing the TtgR regulatory system exhibited a homogeneously induced population over the range of resveratrol concentrations tested (Fig. 2b). The sequencing results of three TtgR mutants are shown in Table S5. A single amino acid substitution, A38T, was found in mu3 protein.

To test the specificity of variant mu3 as an *in vivo* biosensor of resveratrol, the induction effects of the precursors of resveratrol biosynthesis, *p*-coumaric acid and tyrosine, along with an analog caffeic acid, on mu3 were examined. The induction was much higher in response to resveratrol than to the other tested compounds (Fig. 2c). This indicates the applicability of variant mu3 in resveratrol biosynthetic pathway engineering, since its response to resveratrol is not influenced by the presence of precursors.

3.3. Effectiveness of variant mu3 as an in vivo biosensor of resveratrol

Resveratrol biosynthesis in plants occurs *via* a pathway involving phenylalanine ammonia lyase (PAL), cinnamic acid 4-hydroxylase (C4H), 4CL, and stilbene synthase (STS). C4H is poorly expressed in *E. coli* (Limem et al., 2008; Rodrigues et al., 2015), therefore a pathway consisting of tyrosine ammonia lyase (TAL), 4CL, and STS is adopted to synthesize resveratrol (Fig. S1). In our study, 4CL1 from *Arabidopsis thaliana* and STS from *Vitis vinifera* were combined for resveratrol biosynthesis in *E. coli* BW25113 using *p*-coumaric acid as substrate. 4CL1 and STS were cloned downstream of the constitutive GAP promoter (Lim et al., 2011) in two different sequences. The construction in which STS was located upstream of 4CL1 produced a higher level of resveratrol, which was verified by MS (Fig. S5). The biosynthetic pathway was co-expressed with variant mu3 as well as the $P_{ttgABC2}$-RFP construct. After culturing for 18 h in the presence of 985 mg/L *p*-coumaric acid, cells exhibited significantly enhanced RFP fluorescence as compared to those harboring a control plasmid expressing only 4CL1 or STS alone, or neither (Fig. 3a). We also found that RFP fluorescence was positively correlated with resveratrol production, indicating that RFP expression was induced by endogenous resveratrol produced *via* the biosynthetic pathway (Fig. 3b).

3.4. Engineering 4CL1 enzyme in the resveratrol biosynthetic pathway

Strain M3 was constructed by integrating the constitutively expressed variant mu3 and the $P_{ttgABC2}$-*lacZ* construct into the chromosome of strain BW25113. RFP was replaced with LacZ to allow

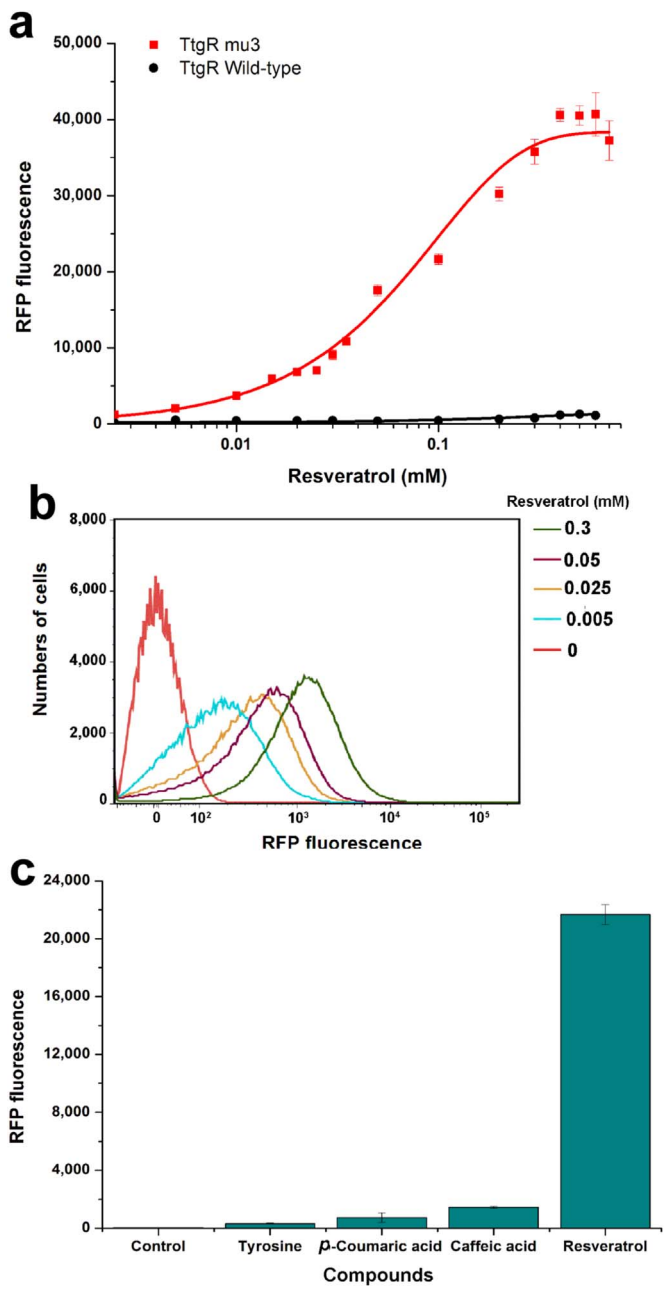

Fig. 2. Characterization of the resveratrol biosensor designed in this study. (a) RFP fluorescence of strain BW25113 expressing the TtgR variant mu3 as a function of resveratrol concentration. The corresponding dose-response curve of wild-type TtgR is included for reference. (b) Flow cytometric analysis of RFP expression in strain BW25113 expressing the TtgR variant mu3 induced by 0 mM (red line), 0.005 mM (blue line), 0.025 mM (yellow line), 0.05 mM (purple line), and 0.3 mM (green line) resveratrol. (c) Induction effects of resveratrol (0.1 mM), tyrosine (6 mM), *p*-coumaric acid (6 mM) or caffeic acid (6 mM) on TtgR variant mu3, compared with Control (in the absence of inducer). The data shown are from three replicate experiments and are expressed as the mean ± standard deviation (SD).

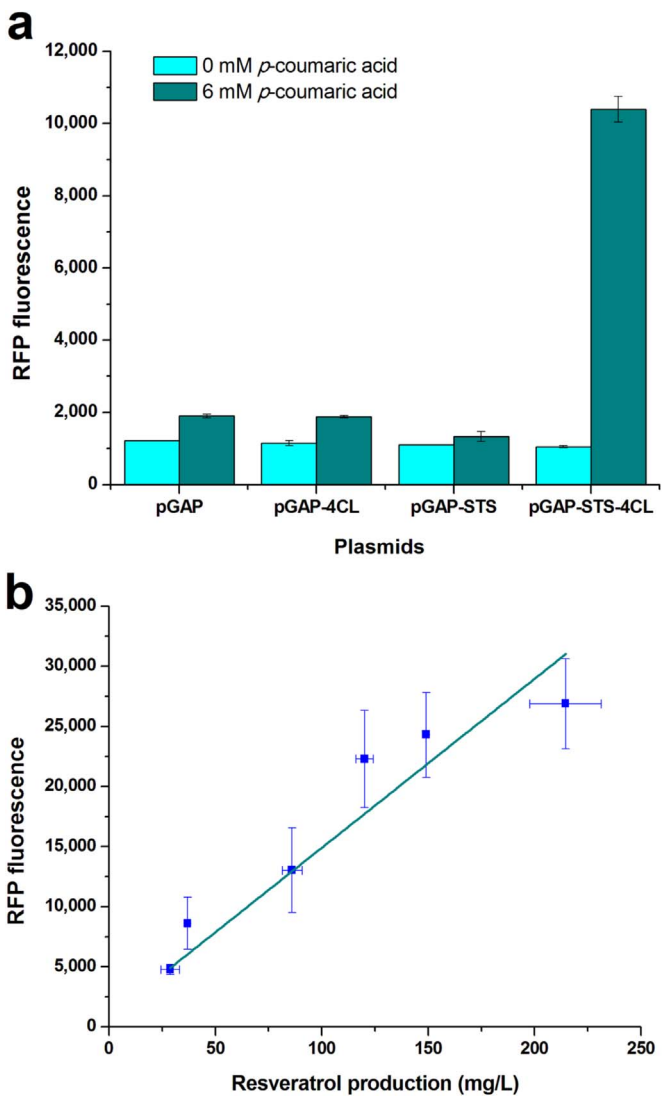

Fig. 3. The biosensor effectively reports the intracellular resveratrol concentrations. (a) RFP fluorescence of strain BW25113 harboring the TtgR variant mu3, the $P_{ttgABC2}$-RFP construct, and a plasmid expressing the complete resveratrol biosynthetic pathway (STS-4CL), 4CL or STS alone, or neither. Strains were cultured for 18 h in the presence of 985 mg/L *p*-coumaric acid. (b) Relationship between RFP fluorescence and resveratrol production by strain BW25113 harboring the TtgR variant mu3, the $P_{ttgABC2}$-RFP construct, and the complete resveratrol biosynthetic pathway. Strains were cultured in the presence of 985 mg/L *p*-coumaric acid at 37 °C. The data shown are from three replicate experiments and are expressed as the mean ± SD.

selection on agar plates. A random mutagenesis library of 4CL1 was constructed, and the resveratrol biosynthetic pathway containing the library was introduced into strain M3 for high-throughput screening (Fig. S6). Three of the darkest blue colonies (identified by the eye) selected from a total of 1.0×10^5 transformants were re-screened by quantifying resveratrol production from the liquid culture of each clone. Clones 2L1, 2E3, and 4AT produced 1.6-, 2.5-, and 4.7-fold higher levels of resveratrol, respectively, than the strain expressing wild-type pathway (Fig. 4a). Purification and re-transformation of the plasmids from these clones into strain M3 confirmed the increase in resveratrol production. Thus, the resveratrol hyper-producers could be rapidly identified from the library by a visual screen. Amino acid substitutions in the selected 4CL1 mutants were confirmed by sequencing (Table S5).

3.5. Activity assay and kinetic studies of wild-type and mutant 4CL1

The genes encoding wild-type 4CL1 and the mutant enzymes were re-cloned into vector pET28a and the resultant N-terminal His-tagged fusion proteins were purified for activity assay and determination of kinetic parameters. The 4AT mutant enzyme exhibited a 1.8-fold increase in specific activity relative to the wild-type enzyme (Fig. 4b). Thus, the improved activity of 4CL1, the first enzyme in the pathway, was the main factor underlying enhanced resveratrol production in the selected hyper-producing strain. Kinetic analyses revealed that the affinity of the mutant enzymes for *p*-coumaric acid was higher than that of wild-type enzyme, which could significantly accelerate the enzymatic

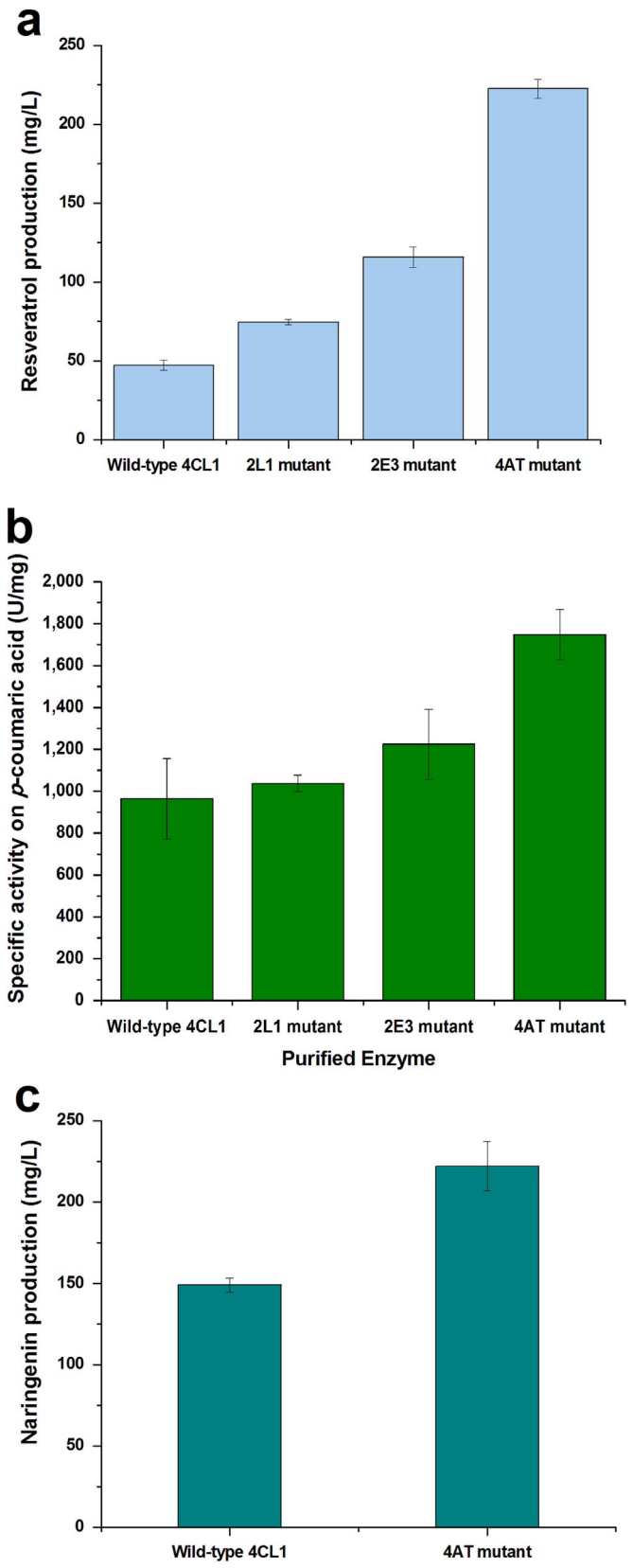

Fig. 4. Screening and characterization of 4CL1 mutants with improved specific activity. (a) Resveratrol production of selected strains expressing 4CL1 variants as compared to the wild-type strain. Strains were cultured for 24 h at 37 °C in the presence of 985 mg/L *p*-coumaric acid. (b) Specific activities of purified wild-type and 4AT mutant enzymes on *p*-coumaric acid. (c) Naringenin productions of strain BW25113 expressing naringenin biosynthetic pathway, including the 4CL1 wild-type or 4AT mutant enzyme. Strains were cultured for 60 h at 30 °C in the presence of 492 mg/L *p*-coumaric acid. The data shown are from three replicate experiments and are expressed as the mean ± SD.

Table 1
Kinetic parameters of the wild-type and mutant 4CL1s on substrate *p*-coumaric acid.

Enzyme	K_m (μM)	k_{cat} (min^{-1})	k_{cat} /K_m ($\mu M^{-1}\ min^{-1}$)
Wild-type 4CL1	50.8 ± 6.6	160.8 ± 1.2	3.2
2L1 mutant	6.5 ± 0.34	22.2 ± 0.62	3.4
2E3 mutant	3.7 ± 0.79	14.9 ± 0.39	4.0
4AT mutant	13.8 ± 1.4	74.2 ± 3.7	5.3

The data shown are from three replicate experiments and are expressed as the mean ± SD.

reaction when the intracellular *p*-coumaric acid concentration is low. The catalytic efficiency (k_{cat}/K_m) on *p*-coumaric acid was 1.7-fold higher for the 4AT mutant than for the wild-type enzyme (Table 1).

3.6. Time course analysis of cells expressing wild-type or mutant 4CL1

The time courses of RFP fluorescence and cell growth for strain BW25113 co-expressing variant mu3, $P_{ttgABC2}$-RFP construct, and resveratrol biosynthetic pathway containing variant 4AT or wild-type 4CL1 were analyzed (Fig. 5). The growth rate and RFP fluorescence were higher in the 4AT- as compared to the 4CL1-expressing strain. Since *p*-coumaric acid is reported to inhibit cell growth (Watts et al., 2006), it was speculated that the enhanced activity of 4AT resulted in the more rapid conversion of *p*-coumaric acid to *p*-coumaroyl-CoA as compared to 4CL1 in the early stages, which reduced the cell growth inhibition and further accelerated the conversion of *p*-coumaric acid to resveratrol.

3.7. Identification of a resveratrol biosynthesis byproduct

A byproduct was detected in cultures of cells expressing the resveratrol biosynthetic pathway (Fig. S7a). To investigate its formation, purified 4CL1 and STS were combined or individually reacted with the substrates *p*-coumaric acid, ATP, CoA, and malonyl-CoA. The byproduct was detected only in the presence of both enzymes, indicating that it was formed by STS with *p*-coumaroyl-CoA as a substrate (Fig. S8). The molecular formula of the byproduct was assigned as $C_{13}H_{10}O_4$ by the positive-mode HRESIMS (*m/z* 231.0670 $[M+H]^+$, calcd for $C_{13}H_{11}O_4$, 231.0652) (Fig. S7b). On the basis of 1D and 2D NMR data (Table S6), the byproduct was identified to be bisnoryangonin which is formed by condensation of *p*-coumaroyl-CoA and two molecules of malonyl-CoA (Fig. S7c).

Bisnoryangonin has been reported to be produced by a type III polyketide synthase from *Oryza sativa* (Go et al., 2015). The formation of bisnoryangonin in this study suggested an inadequate cellular supply of malonyl-CoA for resveratrol synthesis. The amount of accumulated bisnoryangonin was markedly higher in cultures expressing variant 4AT as compared to 4CL1 (Fig. S9). Addition of 0.8 mg/L triclosan, an inhibitor of fatty acid biosynthesis that leads to increased level of intracellular malonyl-CoA, reduced the accumulation of bisnoryangonin by 11.4% and improved resveratrol production by 48.0% (Fig. S9). This provides collateral evidence that bisnoryangonin formation could be due to insufficient malonyl-CoA availability and suggesting that increasing the level of malonyl-CoA could favor resveratrol formation. Given that the levels of both resveratrol and bisnoryangonin were significantly higher in the strain harboring 4AT, engineering the host cell for higher level of endogenous malonyl-CoA was expected to further improve resveratrol production. Under shaking culture conditions, strain M3 expressing 4AT and STS produced 556.4 ± 5.6 mg/L resveratrol in liquid culture, in the presence of 0.8 mg/L triclosan.

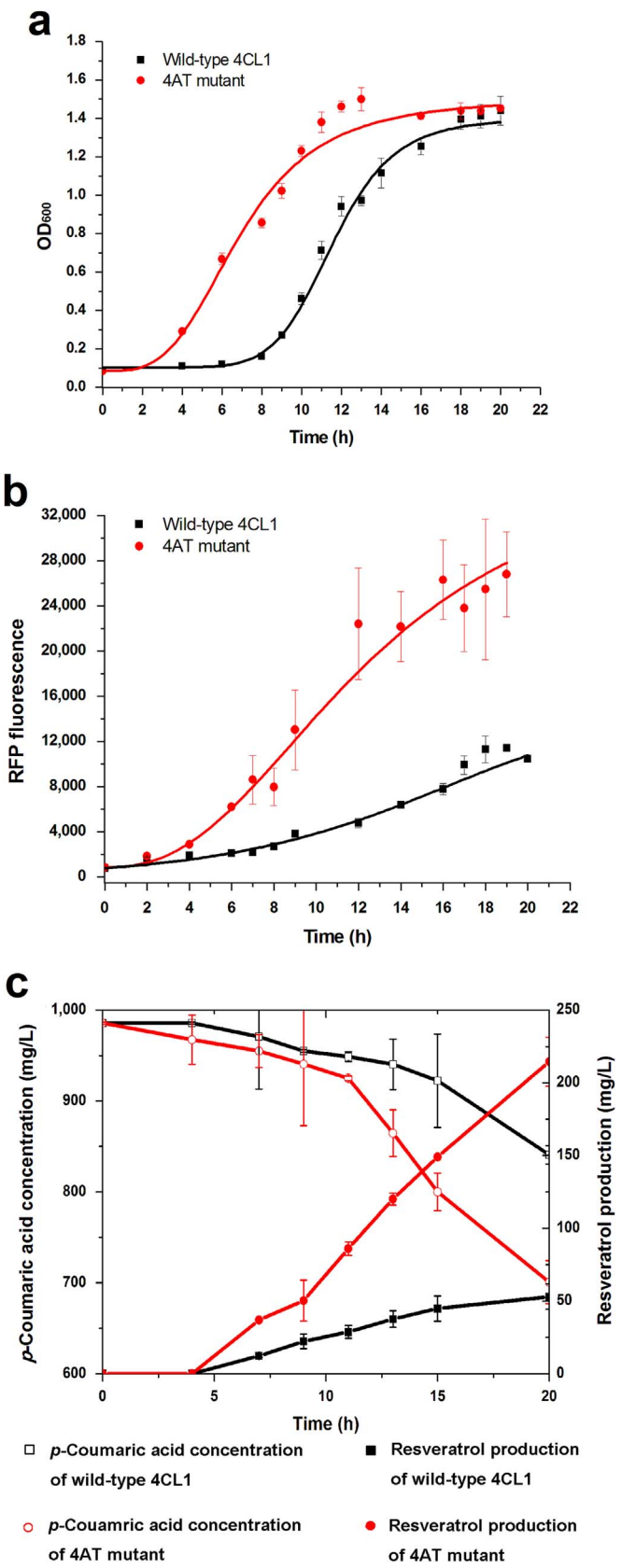

Fig. 5. The time courses of cell growth (a), RFP fluorescence (b), substrate consumption and resveratrol production (c) of strain BW25113 co-expressing variant mu3, $P_{ttgABC2}$-RFP construct and the resveratrol biosynthetic pathway containing 4AT mutant enzyme (circle) or wild-type 4CL1 (square). The strains were cultured in the presence of 985 mg/L *p*-coumaric acid at 37 °C. The data shown are from three replicate experiments and are expressed as the mean ± SD.

3.8. Application of the 4AT mutant in flavonoid biosynthesis

4CL1 catalyzes the conversion of *p*-coumaric acid to *p*-coumaroyl-CoA which is the precursor for synthesis of not only resveratrol but also flavonoids and curcumin derivatives (Fig. S1). Subsequently, the improved 4CL1 variants are potentially applicable to the biosynthesis of a variety of aromatic compounds. To verify this, the 4AT mutant and wild-type 4CL were individually combined with chalcone synthase from *Petunia x hybrida* to construct a pathway for the biosynthesis of naringenin chalcone, which was then spontaneously converted to naringenin (Fig. S1). The formation of naringenin was verified (Fig. S10) and its production was 49.1% higher in the strain expressing 4AT as compared to that expressing 4CL1 (Fig. 4c).

4. Discussion

Key performance features for high-quality transcription factor-based biosensors include specificity, response range, and robustness. The specificity of a biosensor can be assessed by its negative response towards molecules other than the target, which prevents non-specific reporter gene expression. A broad response range facilitates sensitive detection of the target molecule. Finally, the robustness of a biosensor allows stable performance under various conditions, especially in the presence of additional metabolic burden of host cells triggered by heterologous pathway expression, extra energy and cofactors requirement for biosynthetic reactions or high concentrations of biosynthetic precursors or products.

No reports are available to date regarding effector specificity alteration of TtgR. In this study, TtgR was engineered into a resveratrol biosensor, and its effectiveness was verified by the selection of 4CL variants with improved catalytic efficiency. The biosensor has been demonstrated exhibiting specific response towards resveratrol, but not to the biosynthetic precursors *p*-coumaric acid and tyrosine. Other CoA derivative precursors in resveratrol biosynthesis did not induce the biosensor due to their bulky structures. The TtgR variant mu3 exhibited 363-fold induction over a 0–0.6 mM resveratrol range, enabling highly sensitive selection of resveratrol hyper-producers during the biosynthetic pathway engineering. The response of the biosensor was maintained irrespective of the metabolic burden brought about by co-expression with resveratrol biosynthetic pathway in the presence of relatively high concentrations of *p*-coumaric acid, indicating good stability.

4CL1 from *Arabidopsis thaliana* exhibited catalytic activity on *p*-coumaric acid, caffeic acid, cinnamic acid and ferulic acid (Ehlting et al., 1999), and its crystal structure information is unknown. The apo structure of fusion protein comprising of *A. thaliana* 4CL1 and *V. vinifera* STS has been reported by Wang et al. (Wang et al., 2011a, 2011b), while only two 4CL structures from other sources (Hu et al., 2010; Li and Nair, 2015) have been previously reported. Resveratrol is a stilbene phytoalexin found in many plants which has been reported to possess antioxidant (Duan et al., 2016), antiviral (Zhao et al., 2016), and antitumor (Athar et al., 2007; Harikumar et al., 2015) activities and used in the pharmaceutical, health, and cosmetics industries (Abu-Amero et al., 2016; Singh and Pai, 2014; Zhu et al., 2015). Resveratrol has been heterogeneously produced by engineered *Saccharomyces cerevisiae* (Li et al., 2015; Wang et al., 2011a, 2011b) and *E. coli* (Lim et al., 2011; Wang et al., 2015). The resveratrol biosensor developed in this study allows rapid and sensitive screening of resveratrol hyper-producers among strains expressing the 4CL1 mutagenesis library. The 4AT mutant enzyme with enhanced activity thus identified displayed a 4.7-fold improved production of resveratrol, and the simultaneous increased accumulation of the byproduct bisnoryangonin indicated that the resveratrol production can be further improved with a higher supply of malonyl-CoA, which is substrate for the second enzyme of the pathway. The results from kinetic and time-course studies (Table 1, Fig. 5) indicated that a more rapid conversion

of *p*-coumaric acid to coumaroyl-CoA by the 4AT mutant in the early stages reduced the cell growth inhibition and led to improved resveratrol production. Thus, the results from this study highlighted the advantage of *in vivo* directed evolution strategy for pathway enzyme engineering, compared with rational design, since the mutant enzymes overcoming the specific *in vivo* limiting factors can be selected. Although the affinity of 4AT for *p*-coumaric acid was increased, the amino acid substitutions were distant from the binding pocket of this substrate, revealed by analyses using the 4CL structures from *Populus tomentosa* (Hu et al., 2010) or *Nicotiana tabacum* (Li and Nair, 2015).

The 4AT mutant enzyme was incorporated into the biosynthetic pathway of the flavonoid naringenin and found to improve the biosynthesis efficiency of naringenin. Thus the 4AT mutant has potential applications in the overproduction of more natural products. These results demonstrate the feasibility of engineering the key enzyme involving multiple biosynthetic pathways using a designed specialized *in vivo* biosensor for one of the pathway products. A similar strategy could be applied to screen high-activity variants of TAL, another enzyme involved in multiple biosynthetic pathways for a variety of tyrosine-derived aromatic compounds, since the resveratrol biosensor also showed a negative response to tyrosine, the substrate of TAL. Furthermore, as an efficient detection system for endogenous resveratrol, the TtgR variant mu3 can also be used to engineer the host cell genome to further improve resveratrol production. In addition to the use as an *in vivo* biosensor of resveratrol, as a stringently controlled regulatory system with a 363-fold induction dynamic range, this novel gene switch can also be used to fine-tune metabolic pathway flux and control virulent protein expression.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ymben.2017.01.006.

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