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ORIGINAL PAPER

A novel process for obtaining pinosylvin using combinatorial bioengineering in *Escherichia coli*

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Abstract Pinosylvin as a bioactive stilbene is of great interest for food supplements and pharmaceuticals development. In comparison to conventional extraction of pinosylvin from plant sources, biosynthesis engineering of microbial cell factories is a sustainable and flexible alternative method. Current synthetic strategies often require expensive phenylpropanoic precursor and inducer, which are not available for large-scale fermentation process. In this study, three bioengineering strategies were described to the development of a simple and economical process for pinosylvin biosynthesis *in Escherichia coli*. Firstly, we evaluated different construct environments to give a highly efficient constitutive system for enzymes of pinosylvin pathway expression: 4-coumarate: coenzyme A ligase (4CL) and stilbene synthase (STS). Secondly, malonyl coenzyme A (malonyl-CoA) is a key precursor of pinosylvin bioproduction and at low level in *E. coli* cell. Thus clustered regularly interspaced short palindromic repeats interference (CRISPRi) was explored to inactivate malonyl-CoA consumption pathway to increase its availability. The resulting pinosylvin content in engineered *E. coli* was obtained a 1.9-fold increase depending on the repression of *fabD* (encoding malonyl-CoA-ACP transacylase) gene. Eventually, a phenylalanine over-producing *E. coli* consisting phenylalanine ammonia lyase was introduced to produce the precursor of pinosylvin, *trans*-cinnamic acid, the crude extraction of cultural medium was used as supplementation for pinosylvin bioproduction. Using these combinatorial processes, 47.49 mg/L pinosylvin was produced from glycerol.

Keywords Pinosylvin · CRISPRi · Biosynthesis · Combinatorial bioengineering

Introduction

Pinosylvin is a natural secondary metabolite that is biosynthesized through a general phenylalanine metabolism pathway in genus *Pinus* (Rivière et al. 2012). Recent studies have exhibited that pinosylvin possessed benefits for human health, including anti-antioxidant, anti-cancer, cardioprotection, anti-inflammation, and other activities (Jancinova et al. 2012; Park et al. 2012; Park et al. 2013; Yeo et al. 2013; Koskela et al. 2014; Laavola et al. 2015). However, pinosylvin is hard to vast purification via phytoextraction because of low concentrations in plants and various isomers or similar compounds were need to separation (Conde et al. 2014). Biosynthesis provides an alternative approach to pinosylvin production. The engineering strategy for the biosynthetic pathway of pinosylvin in *E. coli* cell is shown in Fig. 1a. This strategy involves embedding phenylalanine ammonia lyase (PAL) to convert endogenous/extrinsic L-phenylalanine into *trans*-cinnamic acid. The *trans*-cinnamic acid is then converted into *trans*-

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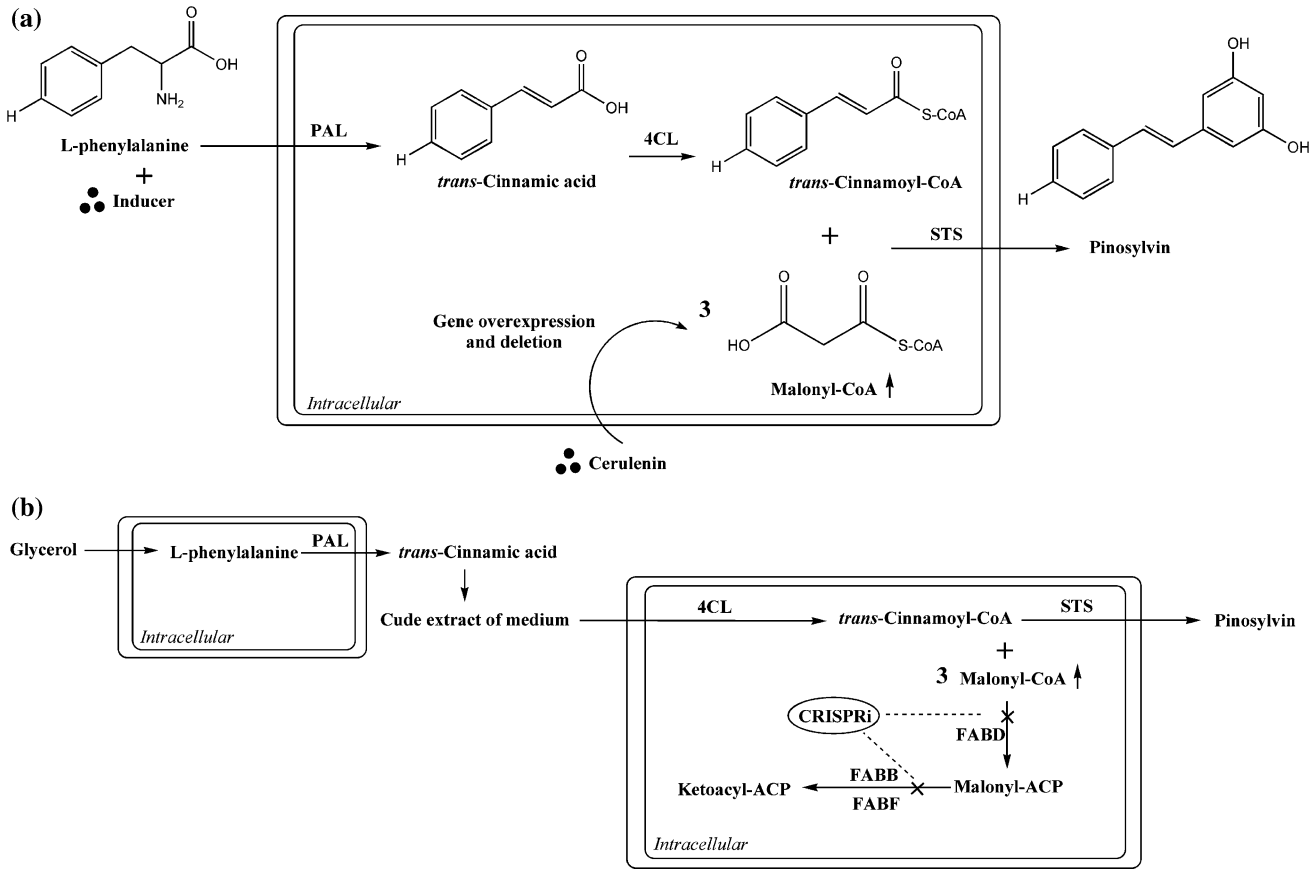

Fig. 1 Engineered pathways for pinosylvin synthesis by recombinant *Escherichia coli*. **a** Strategy in previous studys. *PAL* phenylalanine ammonia lyase; *4CL* 4-coumarate: CoA ligase, *STS* stilbene synthase. **b** Strategy in this study. *FABB* β-ketoacyl-acyl carrier protein synthases I, *FABD* malonyl-CoA-ACP transacylase, *FABF* β-ketoacyl-acyl carrier protein synthases II

cinnamoyl-CoA, through 4-coumarate: CoA ligase (4CL). Stilbene synthase (STS) catalyzes the stepwise condensation of three molecules of intracellular malony-CoA with one molecule of *trans*-cinnamoyl-CoA.

To data, microbial cell factories for pinosylvin bioproduction have been developed but efficient conversion of aromatic amino acids to pinosylvin is the most important limiting factor (Katsuyama et al. 2007; Wang et al. 2015). All of the study required inducer of gene expression and expensive chemicals as precursors, typically L-phenylalanine or *trans*-cinnamic acid in comparison to glycerol. Recently, a feasible strategy for increasing the production of pinosylvin was improving the content of intracellular malonyl-CoA by the addition of the fatty acid production inhibitor cerulenin, the titer of pinosylvin has 20-fold enhancement (van Summeren-Wesenhagen and Marienhagen 2015). However, cerulenin is an expensive inducer and cost prohibitive for a production scale fermentation process in terms of food safety. Therefore, another metabolic regulation strategy was investigated to engineered strains with improved levels of malonyl-CoA. A study was introducing *matB* (encoding malonyl-CoA synthetase) and *matC* (encoding dicarboxylate carrier protein) into *E. coli* to uptake the malonate and to convert it into malonyl-CoA (Leonard et al. 2008; Wu et al. 2013). In addition, the stilbene resveratrol production in *E. coli* showed a 1.6-fold increase through overexpression of *pdh* (encoding pyruvate dehydrogenase multi-enzyme complex), *pgk* (encoding phosphoglycerate kinase), and *gapA* (encoding glyceraldehyde-3-phosphate dehydrogenase) and deletion of *fumC* (encoding fumarase) (Bhan et al. 2013).Whereas the strain cannot bear the burden of multi-gene efficient expression in one or two plasmids. Sometimes, strategy of gene deletion is limited because genes associated with the main metabolic pathway cannot be knocked out. Regulating malonyl-CoA metabolism through synthetic antisense RNAs was reported, a 2.5-fold increase in stilbene resveratrol concentration when *fab*D gene expression is inhibited (Yang et al. 2015). The advantage of this strategy is that metabolic regulation would not influence the growth of strains.

A similar method with antisense RNAs system was Clustered regularly interspaced short palindromic repeats interference (CRISPRi), an emerging gene repression

system, was applied on prokaryotic metabolic engineering in recent studies (Ji et al. 2014; Wu et al. 2014; Lv et al. 2015). Two functional modules have been introduced into *E. coli* for genome regulation without genome editing through flexible CRISPR systems (Qi et al. 2013). A catalytically dead Cas9 mutant (dCas9) acts as a simple RNA-guided DNA-binding complex. A small guide RNA (sgRNA) was coexpressed with target-specific complementary region, Cas9-binding hairpin, and transcription terminator. The silencing effects of dCas9-sgRNA can be induced and reversed as required by using CRISPRi-based knockdown of gene expression. And application of CRISPRi for improving stilbenes production has never been reported.

In this study, a combinatorial bioengineering approach was employed for the production of pinosylvin in *E. coli* directly from glycerol without the addition of expensive inducers and precursors (Fig. 1b). BioBrick assembly and strains optimization were used to increase production ability of pinosylvin in *E. coli*. Application of the CRISPRi system to regulation of gene expression related to prokaryotic fatty acid metabolism, the engineered *E. coli* strain with *fab*D interfered shows a 1.9-fold increase in pinosylvin concentration. For production of *trans*-cinnamic acid supplemented to the medium, an L-phenylalanine over-producing *E. coli* carrying the *pal* gene with constitutive system was employed. Combination of the optimized *E. coli* cells and *trans*-cinnamic acid extract at 37 °C for 12 in shake-flask culture yielded 47.49 mg/L pinosylvin.

Materials and methods

Materials and reagents

E. coli DH5α and other strains (listed in Table 1) were used for plasmid cloning and recombinant molecule production. PrimeSTAR® HS DNA Polymerase, Ex*Taq* Polymerase, pMD18-T, restriction enzymes, T4 DNA ligase and Anhydrotetracycline were purchased from Takara Biochemicals Inc (Japan). Plasmid pQE-30 was purchased from Qiagen Inc (Germany). Plasmid pdCas9_bacteria and pgRNA_bacteria was purchased from Addgene Inc (USA). Plasmid DNA was prepared from stock strains by using a Tiangen plasmid miniprep kit (China), while fragment DNA was isolated through gel extraction by a Tiangen gel DNA recovery kit (China). Total RNA was isolated by using the Tiangen TRNzol Universal Reagent Kit (China). The Tiangen Fastquant RT Kit (China) was used to synthesize the cDNA for mRNA analysis. Real-time PCR (RT-PCR) was carried out for mRNA analysis with Tiangen SuperReal PreMix Plus (SYBR Green) (China). The *trans*-cinnamic acid and pinosylvin standards were purchased from Sigma-Aldrich (USA).

Genetic constructs

The constitutive *gap* promoter (the endogenic promoter of glyceraldehyde-3-phosphate dehydrogenase gene in *E. coli*) was cloned from pMD18-pGAP. The *rrnB* T1 terminator was cloned from pQE-30. The *At4cl* fragment (Accession code: NM_104046.2) was cloned from pMD18-*At4cl*. The *Vvsts* fragment (Accession code: KC417319.1) was cloned from pMD18-*Vvsts*. The total expression fragment (fusion of three or four DNA fragments) was amplified though overlap extension PCR. Different sequences of ribosome binding site (RBS) or additional nucleotide sequence were added by specific primers. The total sgRNA expression fragment (two DNA fragments fusion) was amplified by overlap extension PCR.

All constructed plasmids were verified by both colony PCR and sequencing. All primers, RBS sequences (Ribosome Binding Site), PAM sequences (Protospacer Adjacent Motif, sequence: NGG) for sgRNA design and additional sequences were listed in Supplementary data Table 1. All plasmids used in this study are listed in Table 1.

Preparation of *trans*-cinnamic acid extraction

The cells of *E. coli* ATCC31884 harboring pQE30 with *Rgpal* (Accession code: KF770992) was pre-cultured overnight at 37 °C in 10 mL of LB medium along with 100 μg/mL ampicillin antibiotics. Pre-inoculums were diluted into 100 mL of fresh YM9 medium (1xM9 salts, 10 g/L yeast extract, 5 % glycerol, and 42 g/L Morpholinepropanesulfonic acid, pH 7.0) with 100 μg/mL ampicillin antibiotics according to the 1 % volume fraction of bacteria liquid. Shake-flask culture was performed at 200 rpm and 37 °C. Sample was centrifuged at 12,000 rpm (micro liter rotor size: 24 × 2 mL) for 5 min, and the supernatants were extracted with twice volume of ethyl acetate after 12 h of culture. The organic phase was volatilized by rotary evaporator at 40 °C. The sediments were dissolved in 2 mL Tris–HCl (1 mM, pH 9.5) for preparation of pinosylvin synthesis.

Pinosylvin bioproduction through engineered *E. coli*

The culture medium consisted of YM9 medium along with relevant antibiotics. Recombinant *E. coli* cells containing different expression vector were pre-cultured overnight at 37 °C in 100 mL LB medium. Pre-inoculums were collected by centrifugation and resuspended into 100 mL of fresh YM9 medium. For dCAS9 expression, 1 μM of aTc (Anhydrotetracycline) was supplemented at this time.

Table 1 Plasmids and bacterial strains used in this study

Plasmids/strains	Characteristics	Source
Plasmids		
pMD18	mpMB1 replication region; Amp^R	Takara Biochemicals Inc.
pQE-30	pBR322 replication region; Amp^R	Qiagen Inc.
pMD18-*pgap*	pMD18 with *pgap*	Bioengineering Laboratory stocks
pMD18-*At4cl*	pMD18 with *At4cl*	Bioengineering Laboratory stocks
pMD18-*Vvrs*	pMD18 with *Vvrs*	Bioengineering Laboratory stocks
pA1	pMD18 + *Vvrs* + *At4cl* with RBS1 in an operon	This study
pA2	pQE-30 + *Vvrs* + *At4cl* with RBS1 in an operon	This study
pA3	pQE-30 + *Vvrs* + *At4cl* with RBS2 in an operon	This study
pA4	pQE-30 + *Vvrs* + *At4cl* with RBS3 in an operon	This study
pA5	pQE-30 + *Vvrs* + *Nco*I spacer + *At4cl* with RBS2 in an operon	This study
pA6	pQE-30 + *Vvrs* + 31 bp spacer + *At4cl* with RBS2 in an operon	This study
pA7	pQE-30 + *Vvrs* + *At4cl* with RBS2 in two opposite direction of individual operon	This study
pA8	pQE-30 + *Vvrs* + *At4cl* with RBS2 in two same direction of individual operon	This study
pdCas9_bacteria	P15A replication region; $Cam^{R;}$ aTc-inducible expression of a catalytically inactive bacterial Cas9 (dCAS9)	Addgene Inc.
pgRNA_bacteria	pUC19 with expression of customizable guide RNA (gRNA)	Addgene Inc.
pB1	pA7 + gRNA for anti-*fabB*	This study
pB2	pA7 + gRNA for anti-*fabD*	This study
pB3	pA7 + gRNA for anti-*fabF*	This study
pD1	pQE-30 + *Rgpal* with RBS2 in GAP promoter	Laboratory preservation
Strains		
E. coli DH5α	F^-, *φ80lacZΔM15, Δ(lacZYA-argF) U169, deoR, recA1, endA1, hsdR17* ($r_k^- m_k^+$), *phoA, supE44, λ*$^-$, *thi-1, gyrA96, relA1*	Laboratory preservation
E. coli BW27784	F^-,*Δ(araD-araB)567, ΔlacZ4787(::rrnB-3), λ*$^-$, *Δ(araH-araF)570(::FRT), ΔaraEp-532::FRT, φPcp18araE533, Δ(rhaD-rhaB)568, hsdR514*	*E. coli* Genetic Stock Center, New Haven, CT
E. coli MG1665	F^-, *λ*$^-$, *ilvG*$^-$, *rfb-50, rph-1*, "wild type" K-12 strain	*E. coli* Genetic Stock Center, New Haven, CT
E. coli BL21(DE3)	F^-,*ompT, gal, dcm, lon, hsdSB(rB*$^-$ *mB*$^-$*)*, λ(DE3 [*lacI, lacUV5*-T7, *gene1, ind1, sam7, nin5*])	Novagen
E. coli MC1061	*hsdR2, hsdM*$^+$, *hsdS*$^+$, *araD139 Δ(ara-leu)7697, Δ(lac)X74, galE15, galK16, rpsL (StrR), mcrA, mcrB1*	Laboratory preservation
E. coli ATCC31884	*aroH367, tyrR366, tna-2, lacY5, aroF394*^{fbr}, *malT384, pheA101*^{fbr}, *pheO352, aroG397*^{fbr}	American Type Culture Collection
E. coli M15	Host strain for QIA expression system	Qiagen Inc.
P0	*E. coli* BW27784 with pQE-30	This study
P1	*E. coli* BW27784 with pA1	This study
P2	*E. coli* BW27784 with pA2	This study
P3	*E. coli* BW27784 with pA3	This study
P4	*E. coli* BW27784 with pA4	This study
P5	*E. coli* BW27784 with pA5	This study
P6	*E. coli* BW27784 with pA6	This study
P7	*E. coli* BW27784 with pA7	This study
P8	*E. coli* BW27784 with pA8	This study
C0	*E. coli* DH5α co-transformed with pA7 and pdCAS9_bacteria	This study
C1	*E. coli* DH5α co-transformed with pB1 and pdCAS9_bacteria	This study

Table 1 continued

Plasmids/strains	Characteristics	Source
C2	*E. coli* DH5α co-transformed with pB2 and pdCAS9_bacteria	This study
C3	*E. coli* DH5α co-transformed with pB3 and pdCAS9_bacteria	This study
D1	*E. coli* ATCC31884 with pD1	Laboratory preservation

Shake flask fermentation were performed at 200 rpm, 37 °C additionally supplemented with *trans*-cinnamic acid extraction. Samples were taken every few hours and used for the analysis of the cell growth, product accumulation and gene expression.

Expression analysis by real-time PCR

The gene expression levels of *fab*B, *fab*D, *fab*F were analyzed using a Bio-Rad CFX96 real-time PCR detection system (USA). 16S rRNA (Accession code: J01859.1) as the inner standard. Primers were designed from the full-length cDNA sequences (listed in Supplementary data Table 1). Real-time PCR was performed in a 25 μL reaction volume with 0.3 μM of each primer. PCR protocols were as follows: 1 cycle of 15 min at 95 °C; 40 cycles with a denaturing time of 10 s at 94 °C, an annealing time of 20 s at 60 °C, and an elongation time of 20 s at 72 °C. All samples were prepared with three parallel groups to obtain results of ΔCt values from the outputs of RT-PCR.

HPLC analysis of pinosylvin and *trans*-cinnamic acid

All samples were extracted with twice volume of ethyl acetate. The mixture was centrifuged at 12,000 rpm (micro liter rotor size: 24 × 2 mL) for 5 min and the supernatants were analyzed on an HPLC system with a phenomenex ODS reverse phase C-18 column (5 μm, 250 × 4.6 mm) and LC2030 UV Detector. The solvent profile was acetonitrile (buffer A) and water (buffer B) as the mobile phases with a flow rate of 1.0 mL per minute. The injection volume was 20 μL. For pinosylvin detection, samples were separated with the mobile phase composition profile fixed at 40 % buffer A and 60 % buffer B. The characteristic UV absorption spectrum was 294 nm. The metabolites were confirmed by the retention time compared to those of authentic standards which retention time was 12.32 min. *trans*-cinnamic acid was detected under the mobile phase composition profile fixed at 40 % buffer A and 60 % buffer B (0.1 % acetic acid). The characteristic UV absorption spectrum was 272 nm and the retention time was 5.56 min.

Results

Identifying the best constitutive BioBrick combination for pinosylvin bioproduction

RBS is an effective control element for translation and thereby protein expression in *E. coli* (Qi et al. 2012). The replication region determines the copy number of vector as well as the expression level of heterogeneous genes. The high-copy plasmid does not always have maximum gene expression because of metabolic burden effects (Jones et al. 2000). The BioBrick assembly contains a high-copy number plasmid background with a constitutive *gap* promoter operon, and was suitable for stilbene resveratrol pathway assembly in *E. coli* BW27784 (Lim et al. 2011). In this study, we used pGAP, *Vvsts*, and *At4cl* to reconstruct different constitutive plasmids to further study BioBricks combinatorial optimization.

To determine the best RBS and replication region assembly, four plasmids with different RBS and replication regions (Fig. 2a) were transferred into *E. coli* BW27784 for shake flask cultivation supplemented with 0.5 mM *trans*-cinnamic acid. When analyzing the supernatant of fermentation broth by HPLC, a peak was detected at the same retention time of pinosylvin and *trans*-cinnamic acid standard (Supporting Information Fig. 1). With the same RBS, the production with moderate copy number plasmid was approximately sixfold greater than that with the high copy number plasmid (comparison between strains P1 and P2) because the high copy number of vector was not suitable for protein functional folding and plasmid stability (Jones et al. 2000). The production with RBS3 plasmid was approximately 1.16-fold greater than that with the RBS1 plasmid (comparison between strain P4 and P2 at the same replication region) because the strong RBS of pET system took advantage of ribosome binding and transcriptional initiation (Holleley and Geerts 2009). However, no product was detected in strain P3 with RBS2 sequence from reference construct RL027A (Lentini et al. 2013). Therefore, different BioBrick compositions directly influenced the titers of metabolic production. RBS3 with moderate-copy number constructs was used for subsequent experiments.

We then observed pinosylvin production from different configurations of polycistronic transcription assembly. The

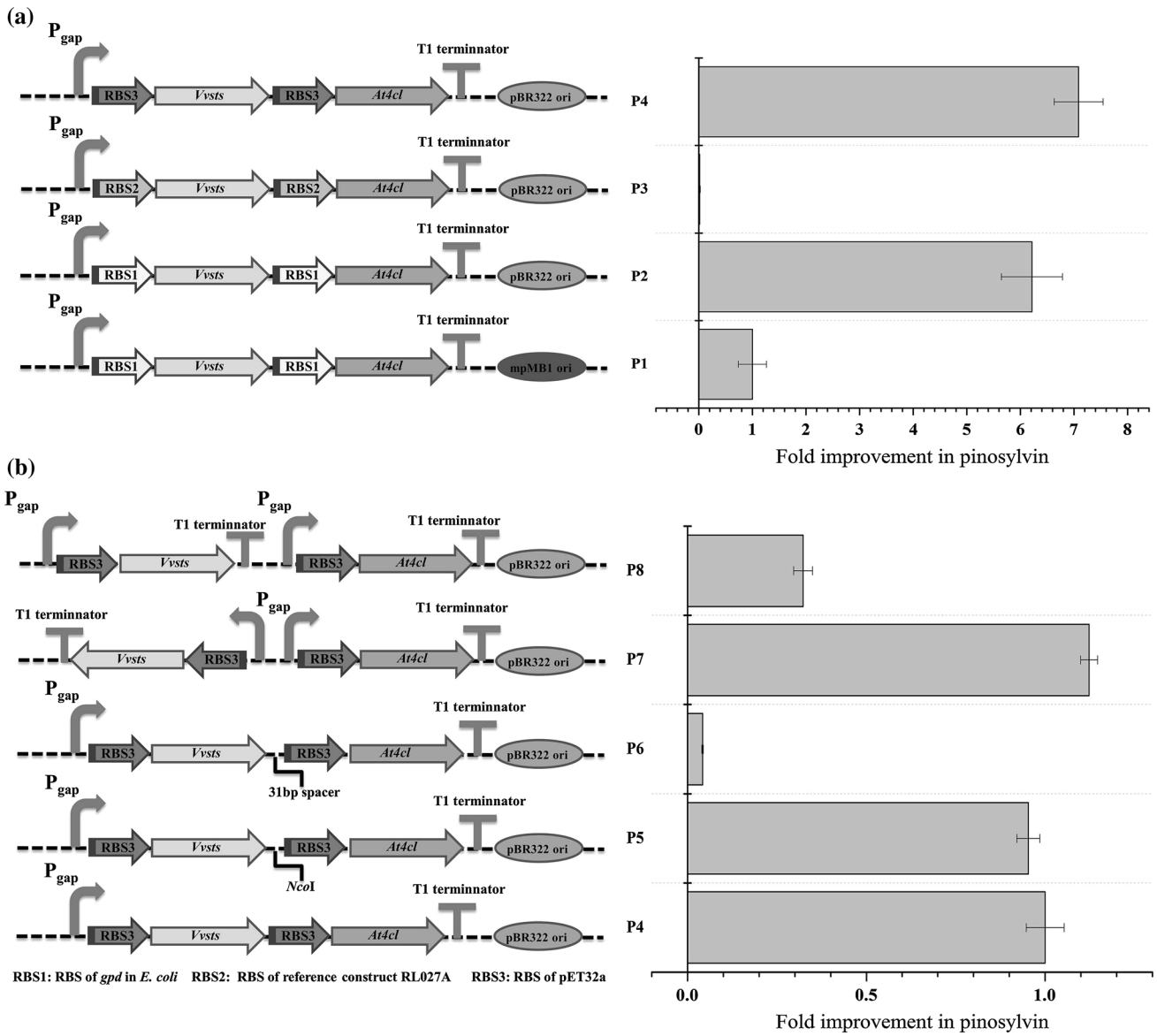

Fig. 2 Pinosylvin bioproduction in *E. coli* containing different constitutive biobrick combination. **a** The effect of configuration of four plasmids with different RBS and replication region. Data are plotted relative to strain P1. **b** The effect of configuration of four plasmids with different polycistron transcriptional assembly. Data are plotted relative to strain P4

influence of upstream sequence composition of RBS on production levels is shown in Fig. 2b. The constructs with no spacer (strain P4) and with a *Nco*I (8 bp) spacer (strain P5) extra restriction enzyme cutting site both resulted in higher relative production titers when compared with the constructs with a 31 bp spacer (strain P6), which was the optimal sequence between two continuous heterogeneous gene expressions from the reference construct RL027A (Lentini et al. 2013). We aimed to answer the following question: which combination of two gene fragments was better for bioproduction. Through the comparison of the strains P4, P7, and P8, the highest pinosylvin production was obtained in *E. coli* BW27784 (strain P7) with the double-promoter construct in opposite expression direction. These results were similar to the exploration of optimal plasmid configuration (Huang et al. 2013).

Determining the best strain for pinosylvin biosynthesis

We examined the plasmid pA7 in seven general lab strains (Table 1) and strain-to-strain variations in pinosylvin titer (Fig. 3) to determine the ideal strain for pinosylvin production. The maximum pinosylvin production was approximately threefold higher than the minimum. *E. coli* Dh5α with pA7 produced the highest yield of pinosylvin

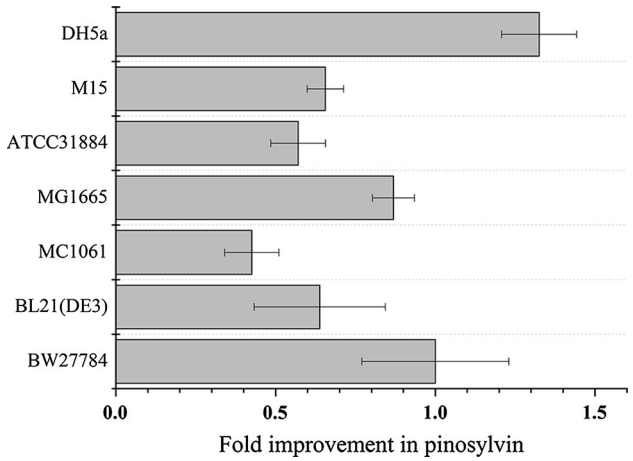

Fig. 3 Comparison of different strains for pinosylvin biosynthesis. Data are plotted relative to *E. coli* BW27784

among the strains. The pinosylvin biosynthetic capacity differed among strains. *E. coli* Dh5α cells displayed preferable metabolic capability and were used for subsequent experiments.

Effect of gene repression with CRISPRi on pinosylvin production

In the fatty acid biosynthesis, the malonyl-CoA: ACP transacylase encoded by *fabD* was a key gene in malonyl-CoA consumption, and the strain growth was limited without it (Janßen and Steinbüchel 2014). The *fabB* and *fabF* (encoding β-ketoacyl-acyl carrier protein synthases [KAS] I and II) gene products were the suppressive target of cerulenin (Leonard et al. 2008; Lim et al. 2011). Therefore we selected these *E. coli* chromosomal genes (*fabB*, *fabD* and *fab*F) as target genes to demonstrate the effectiveness of CRISPRi on the repression of fatty acid biosynthesis and enrichment of pinosylvin production. An engineering strain co-expression of the mutated dCas9 protein and different sgRNA Biobrick was constructed (Fig. 4a). Whole sgRNA Biobrick contains four parts: a 36 bp constitutive promoter J_23119, a 43 bp hairpin region for dCas9 protein binding, a 38 bp tracrRNA terminator and a 20 bp DNA based-pairing region, different gene has different pairing region. In addition, the dCAS9 protein encoded by another plasmid pdCas9_bacteria was conveniently induced by 1 μM aTc.

Real-time PCR results indicated that the CRISPRi system functioned at the mRNA level to regulate reduction of *fabB*, *fabD* and *fab*F gene expression (Fig. 4b). The sgRNA anti-*fab*B, anti-*fab*D and anti-*fab*F were able to bind with their respective targets with different efficiencies ranging from 79.9 to 99.5 % repression. Thus, the CRISPRi system was successfully established as a technique for bacterial fatty acid biosynthesis regulation. Compared with the control group strain C0 with non-target sgRNA construct, repressing *fabB* resulted in a 1.5-fold increase in pinosylvin production (Fig. 4c). The highest increase of pinosylvin yield in strain C2 with the anti-*fabD* construct was 1.9-fold that of the control group strain C0. However, for unknown reason, anti-*fab*F in the strain C3 was no obvious effect on production improvement.

Next we have observed the cell dynamics during pinosylvin bioproduction compared with strain C0 (without sgRNA) and strain C2. Obviously, the biomass of the strain C2 was low after induction of 1 μM aTc because of the inhibition of endogenous fatty acid synthesis. After fermentation for 12 h, the inducer was washed from the growth media, and cells started to grow and propagate (Fig. 5a). These results demonstrate that the silencing effects of dCas9-sgRNA for *fabD* can be induced and reversed. Furthermore, this method can be developed into a biological switch for pinosylvin or other stilbene chemical biosynthesis. Finally, we tested the biosynthetic capacity of pinosylvin in engineered strain C2. Different concentrations of *trans*-cinnamic acid, ranging from 0.25 to 4 mM, were fed into the medium. As shown in Fig. 5b, after 12 h of culture, the pinosylvin production was measured by HPLC analysis. The result suggested that 0.5 mM *trans-cinnamic* acid can be converted to ~0.22 mM (47.49 ± 2.57 mg/L) pinosylvin. Addition of substrates failed to increase the production.

Pinosylvin bioproduction from *trans*-cinnamic acid extraction

In previous study, engineered *E. coli* cell for pinosylvin bioproduction also using the isopropyl-β-D-1-thiogalactopyranoside, IPTG for gene expression and low efficient conversion of aromatic amino acids to pinosylvin, up to 13 mg/L production was detected in medium (Katsuyama et al. 2007; van Summeren-Wesenhagen and Marienhagen 2015; Wang et al. 2015). When the engineered strain P7 harboring *Vvsts* and *At4cl* in double-promoter construct with opposite expression direction, higher efficient conversion of *trans*-cinnamic acid to pinosylvin (production was 25.05 ± 2.55 mg/L) was explored. However, the addition of *trans*-cinnamic acid was not suitable for larger fermentation in terms of food safety, because of so many *trans*-cinnamic acid is obtained by chemical synthesis routes by benzaldehyde from petroleum industry. Thus we first examined stepwise for pinosylvin bioproduction. To reduce the cost of substrate, we selected a phenylalanine overproduction *E. coli* ATCC31884, and engineered the strain with PAL expression to *trans*-cinnamic acid production from its phenylalanine (strain D1). After shake-flask culture for 12 h, 165.57 ± 4.85 mg/L *trans*-cinnamic

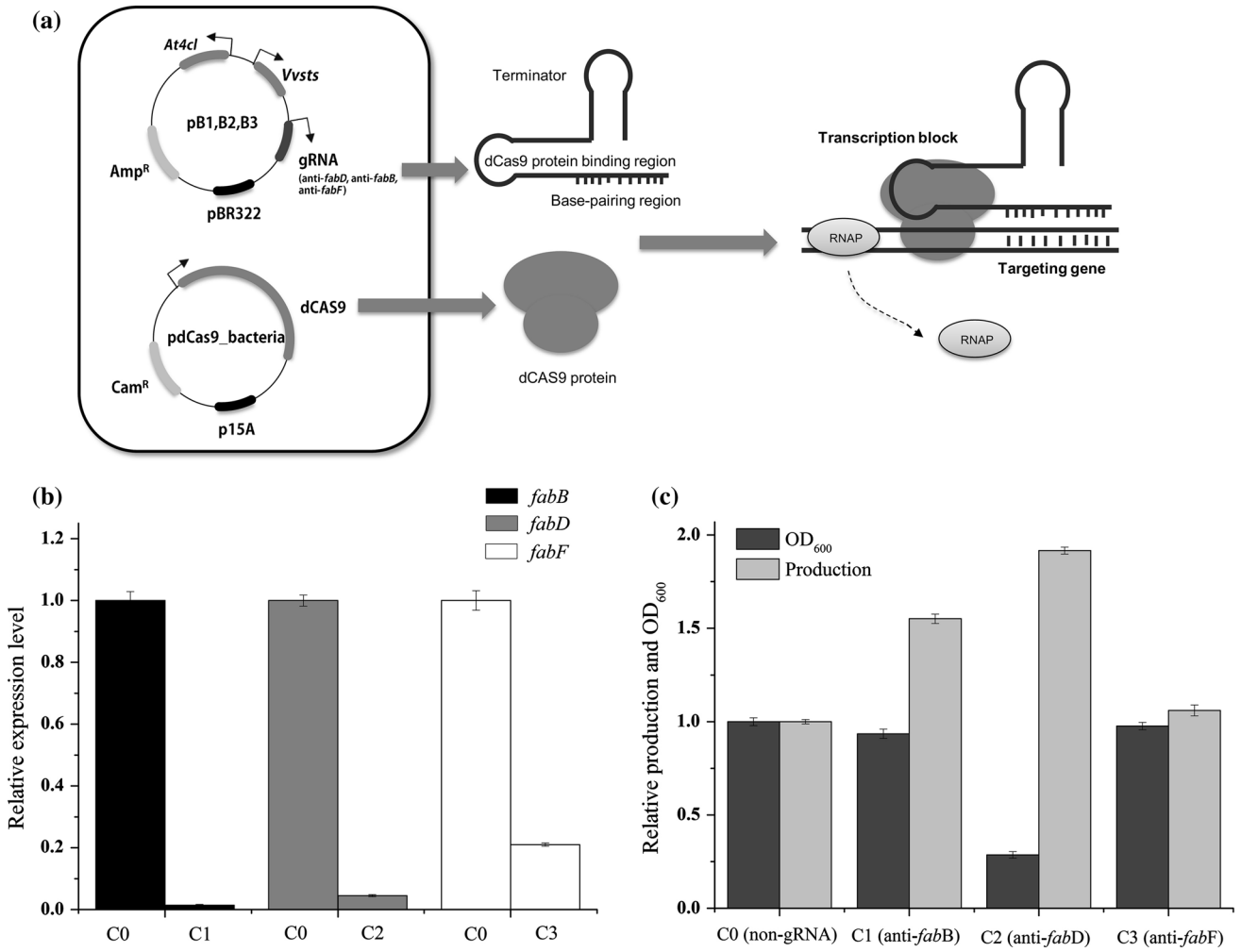

Fig. 4 Metabolic regulation of *E. coli* for pinosylvin bioproduction using CRIPSPi strategy. **a** The plasmids used for dCAS9 expression and sgRNA-based repression. Process of the CRISPRi system to block transcription initiation. **b** Gene expression in recombinant *E. coli*. C0, Control; C1, anti-*fab*B; C2, anti-*fab*D; C3, anti-*fab*F. **c** The effect of configuration of three plasmids with different targeted-sgRNA assembly. Data are plotted relative to strain C0

acid was detected in medium. According to the transformational capacity of *trans*-cinnamic acid (Fig. 5b) in engineered strain C2, the crude extract contained 0.5 mM (74 mg/L) *trans*-cinnamic acid was added into the culture medium of engineered strain C2. Finally, the yield of pinosylvin was approximate 47 mg/L by HPLC detection. These results suggested that we successfully build up a process for pinosylvin biosynthesis from *trans*-cinnamic acid extraction in *Escherichia coli*.

Discussion

Phenylpropanoids, which include stilbenoids, flavonoids, curcuminoids, and cinnamyl anthranilate, are a diverse group of secondary metabolites synthesized from L-phenylalanine or L-tyrosine; these compounds exhibit various bioactivities. Recently, much more study for natural product synthesis by different artificial biological systems (Zhou et al. 2014). For solution to the existing problem about high cost and low conversion efficiency, these work systematically demonstrated that a combinatorial strategy for pin pinosylvin bioproduction.

BioBrick assembly and metabolic regulation are essential to facilitate the construction of microbial cell factories for phenylpropanoid bioproduction. We have successfully improved the biosynthetic capacity of pinosylvin in *E. coli* by BioBrick combinatorial optimization. The engineered *E. coli* Dh5α carrying *Vvsts* and *At4cl* in double GAP promoter construct with opposite expression direction, which displays higher efficient conversion of *trans*-cinnamic acid to pinosylvin.

The CRISPRi system offers the possibility of repressing expression of genes related with fatty acid metabolism,

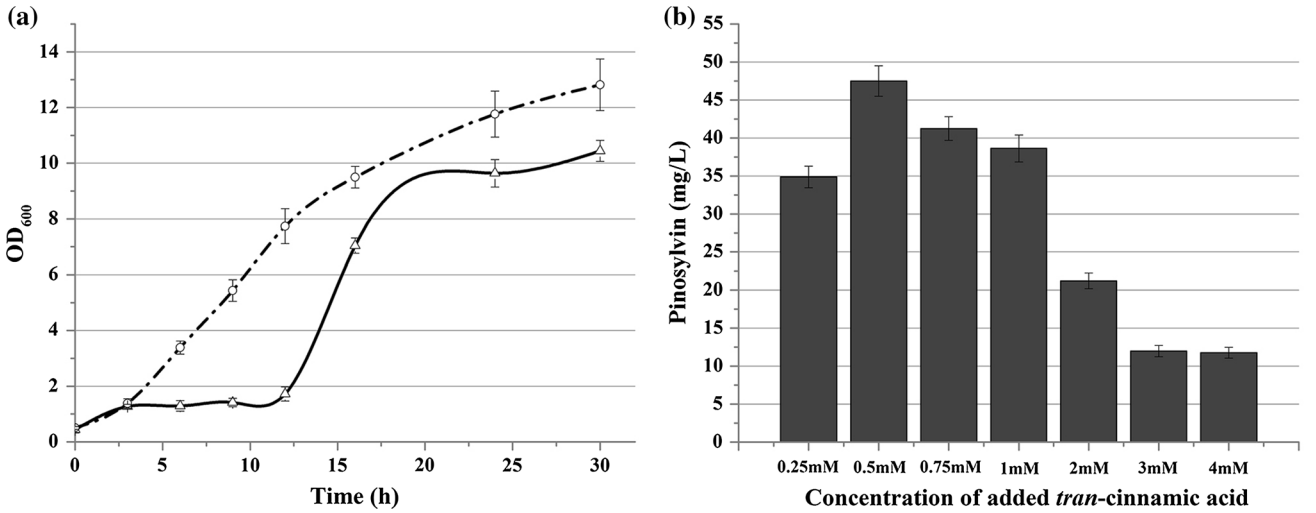

Fig. 5 **a** Cell dynamics of production of pinosylvin with recombinant *E. coli*. *Open circle* strain C0; *open pointed up triangle* strain C2. **b** Effects of *trans*-cinnamic acid concentration on pinosylvin production profile for 12 h of fermentation for strain C2

thus avoiding labor-intensive multiple gene expression and deletion. In addition, CRISPRi avoids the disadvantage of the traditional gene knockout method, especially the *fab*D gene was repressed and not lead to strain death. Just like a function of switch, CRISPRi can be used to repress the target gene any time, and its effects are reversible. Although this improvement of production was less than that obtained when cerulenin was used (van Summeren-Wesenhagen and Marienhagen 2015), this method could be an alternative to the *fab*B and *fab*F gene repression in *E. coli*. The CRISPRi system works to regulate expression of *fab*D gene, a maximum increasement (1.9-fold) of pinosylvin was investigated. To our knowledge, this is the first report to explore the possibility of using CRISPRi system for improving stilbene production in *E. coli*. In comparison to another similar function method, synthetic antisense RNAs, the stages of gene regulatory level in the genome is different. The synthetic antisense RNAs system controls the expression of target genes in *trans* at the post-transcriptional level (Na et al. 2013), while the CRISPRi system influences the gene coding sequence to block transcription initiation or elongation (Qi et al. 2013). So we also want to have experiment to find out a more flexible and economical method further.

Finally, we also demonstrated the feasibility of rational design of stepwise pinosylvin biosynthesis from glycerol. However, the yield was not satisfactory for industrial-scale production. A larger scale fermentation for improving pinosylvin bioproduction will be experiment in the future. The *trans*-cinnamic acid was usually used as the starting precursor to produce phenylpropanoids, such as naringenin, pinocembrin, chrysin and so on (Kong 2015). Thus we believe that these combinatorial processed also could be a sustainable and flexible alternative method for microbial production of phenylpropanoids.

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