Regulation of ATP levels in *Escherichia coli* using CRISPR interference for enhanced pinocembrin production

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**Abstract**

**Background:** Microbial biosynthesis of natural products holds promise for preclinical studies and treating diseases. For instance, pinocembrin is a natural flavonoid with important pharmacologic characteristics and is widely used in preclinical studies. However, high yield of natural products production is often limited by the intracellular cofactor level, including adenosine triphosphate (ATP). To address this challenge, tailored modification of ATP concentration in *Escherichia coli* was applied in efficient pinocembrin production.

**Results:** In the present study, a clustered regularly interspaced short palindromic repeats (CRISPR) interference system was performed for screening several ATP-related candidate genes, where *metK* and *proB* showed its potential to improve ATP level and increased pinocembrin production. Subsequently, the repression efficiency of *metK* and *proB* were optimized to achieve the appropriate levels of ATP and enhancing the pinocembrin production, which allowed the pinocembrin titer increased to 102.02 mg/L. Coupled with the malonyl-CoA engineering and optimization of culture and induction condition, a final pinocembrin titer of 165.31 mg/L was achieved, which is 10.2-fold higher than control strains.

**Conclusions:** Our results introduce a strategy to approach the efficient biosynthesis of pinocembrin via ATP level strengthen using CRISPR interference. Furthermore coupled with the malonyl-CoA engineering and induction condition have been optimized for pinocembrin production. The results and engineering strategies demonstrated here would hold promise for the ATP level improvement of other flavonoids by CRISPRi system, thereby facilitating other flavonoids production.

**Keywords:** Flavonoids, ATP, CRISPRi, Pinocembrin
active only in the presence of energy carriers (ATP) [7]. For example, among the 971 reactions of natural product biosynthesis identified in *Streptomyces coelicolor*, more than 21% rely on the participation of ATP [8]. Also, ATP is required for reconstitution of heterocycle forming activity with the microcin B17 precursor [9]. Previous research suggested that, ATP pathway modification might be an alternative way to enhance the final product biosynthesis. For instance, Zhao found that the increase of ATP supply would improve the production of terpenoids compounds [10].

ATP is synthesized through ATP synthase and electron transfer chain in *E. coli* [10]. In order to increase ATP supply, different gene operons were modulated in *E. coli* with five regulatory parts, lead to 20%, 16%, 5% and 21% increase in β-carotene yield [10]. In addition, the additive of sodium citrate acts as an auxiliary energy substrate for the accumulation of intracellular ATP and the increase of ATP/ADP ratio, lead to the overproduction of S-adenosylmethionine (SAM) and glutathione (GSH) [11]. Although the overexpression of ATPase and optimization of fermentation process are effective ways to improve ATP supply, the repression of the consumption pathway to increase the ATP accumulation is an alternative option [12]. It was reported that the disruption of the ATP consuming bypass pathway in *Saccharomyces cerevisiae* could achieve 3.1-fold higher ATP-generating activity and 1.7-fold higher glutathione productivity compared with the control strain. Furthermore, Chen [13] found that the sRNA-based downregulation strategy in *E. coli* could increase the ATP level and S-adenosylmethionine production. Recently, the clustered regularly interspaced short palindromic repeats interference (CRISPRi) system provides an efficient tool for targeted gene regulation in a sequence-specific manner in *E. coli* [14]. Compared with sRNAs and antisense RNAs strategies [15, 16], this RNA-guided DNA recognition platform provides a simple approach for repression of multiple target genes simultaneously on a genome-wide scale [17–19]. Accordingly, the development of genetic engineering has offered an alternative approach utilizing a CRISPRi regulation strategy to control the intracellular ATP level. In the biosynthesis of flavonoid, ATP has been proved not only to provide adenosyl moiety in *E. coli* metabolism but also to play the role of an energy carrier in pinocembrin biosynthesis. However, there is no ATP engineering strategies for increasing pinocembrin biosynthesis has been proposed to date.

In this study, we demonstrated that the ATP additive was advantageous for pinocembrin production and 5 genes involved in ATP metabolic pathways were repressed by the CRISPRi system to identify individual

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**Fig. 1** Metabolic pathway for pinocembrin production in *E. coli*. In the metabolic map, reactions from the pinocembrin pathway are shown in violet and Up-regulations are shown blue. DAHP, 3-deoxy-o-arabino-heptulosonate-7-phosphate; SAM, S-adenosylmethionine; PAL, phenylalanine ammonia lyase encoded by *pal*; 4CL, 4-coumaroyl-CoA ligase encoded by *4cl*; CHS, chalcone synthase encoded by *chs*; CHI, chalcone isomerase encoded by *chi*.
target genes that could increase the ATP level and pinocembrin production. Furthermore, multiple genes repressing for ATP engineering was performed to achieve the most effective combination. Finally, the pinocembrin production was further improved by combination of ATP and malonyl-CoA engineering. The results and engineering strategies demonstrated here would provide an alternative platform to improve intracellular ATP level in the biosynthesis pathway of natural products.

**Methods**

**Strains and media**

*Escherichia coli* Trans-T1 was used for plasmid propagation. *E. coli* BL21 (DE3) was used to express sgRNA and produce pinocembrin. The compatible vector set (pTrc99a, pCDF303, pRSFDuet-1, and pACYCDuet-1) was used to express multiple genes in one strain. *E. coli* seed cultures were grown in Luria–Bertani (LB) medium. MOPS minimal medium supplemented with 5 g/L glucose and 4 g/L NH₄Cl was used for sgRNA expression and pinocembrin production. MOPS minimal medium contains 10× MOPS mixture 100 mL, 0.132 M K₂HPO₄ 10 mL, milliQ H₂O 880 mL, 1 mg/mL thiamine 0.1 mL. Appropriate antibiotics were added at the following concentrations: 100 μg/mL of ampicillin, 50 μg/mL of kanamycin, 34 μg/mL chloramphenicol and 40 μg/mL streptomycin. Cell growth was detected via the optical density measured at 600 nm (OD₆₀₀) using a spectrophotometer (Thermo Scientific, Waltham, MA, USA). The plasmids and strains used in this study are listed in Tables 1 and 2.

**Construction of sgRNA-expressing plasmids**

pACYC-dCas9 was constructed by amplifying dCas9 fragment from 44249-dCas9 plasmid (no. 44249) (Addgene, USA) into BglII/HindIII sites of pACYCDuet-1. The sgRNA chimera, which consists of five domains [a Trc-inducible promoter, a 20-nucleotide (nt) complementary region for specific DNA binding, a 42-nt dCas9-binding hairpin, a 40-nt transcription terminator derived from *Streptococcus pyogenes* and a 46-nt rrnB transcription terminator] was synthesized by Genewiz (Suzhou, China) and inserted into EcoRI/BamHI sites of pCDF303. This resulted in the plasmid pCDF303-gfp-H, which was the template for PCR-based mutagenesis. Site-directed mutagenesis was performed using the overlap-extension PCR method with mutant-specific primers. Other plasmids for repression were constructed like pCDF303-gfp-H. But the primers are different. Oligo nucleotides used to generate sgRNA cassettes and the resultant sgRNA expression vectors are listed in Additional file 1: Tables S1 and S2. Inhibition of multiple genes is based on the ligation used isocaudomers BglII and BamHI. Plasmid pCDF-proB-L was cut with EcoRI and BglII and plasmid pCDF-metK-H was cut with EcoRI and BamHI. Plasmid pCDF-metK-H-proB-L was obtained after ligation.

**Analytic methods**

To assess the levels of cinnamic acid and pinocembrin, the supernatant was extracted with an equivalent volume of ethyl acetate, vortexed, and centrifuged at 6000 rpm for 3 min at 4 °C. Then, the upper organic layer was removed and evaporated to dryness. The remaining residue was resolubilized with methanol (TEDIA, Fairfield, OH, USA). Samples were quantified by HPLC (Alltech, Deerfield, IL, USA) using an Alltech series 1500 instrument equipped with a prevail C18 reverse-phase column (5 μm, 250 × 4.6 mm; Grace, Deerfield, IL, USA) maintained at 25 °C. For detection, 0.1% acetic acid (solvent A) and acetonitrile supplemented with 0.1% acetic acid (solvent B) were applied as the mobile phases at a flow rate of 1.0 mL min⁻¹. The elution was performed according to the following conditions: minute 0–1: 15% B; minutes 1–10: 15% to 40% B; minutes 10–15: 40% to 50% B; minutes 15–25: 50% to 85% B; minutes 25–30: 85% to 15% B; and minute 30–31: 15% B. Products were detected by monitoring the absorbance at 300 nm.

To quantify the malonyl-CoA and ATP concentration, the harvested cells were resuspended in 1 mL of 6% perchloric acid (ultrasonication in an ice-water bath) and neutralized with 0.3 mL of saturated potassium carbonate. The solution was centrifuged to pellet the cell debris. For detection of malonyl-CoA, 95% 0.1 M ammonium formate/5% MeOH (solvent A) and 50% 0.1 M ammonium formate/50% MeOH (solvent B) were applied as the mobile phases at a flow rate of 1.0 mL min⁻¹. The elution was performed according to the following conditions: minutes 0–10: 100% A to 50% A; minutes 10–12: 50% to 100% A; minutes 12–13: 100% A. For detection of ATP and ADP, the mobile phase used was phosphate buffer consisting of 0.06 M K₂HPO₄ and 0.04 M KH₂PO₄ at pH 7.0 adjusted with 0.1 mol/L KOH and operated at a flow rate of 1 mL/min [13].

**Results**

**The effect of ATP on pinocembrin production**

In the previous study, we constructed the pinocembrin metabolic pathway in *E. coli* BL21 (DE3) by transforming plasmids pTrc-BOPAL-PA4CL and pRSF-CHS-CHI, as a result, a pinocembrin production titer of 14.74 mg/L was achieved [6]. Using this strain as a control check (CK), to ascertain the effect of the ATP on the pinocembrin production, different concentrations of ATP ranging from 0 to 6 mM was added in the medium. As shown in Fig. 2, with an increasing ATP concentration from 0 to 6 mM, the pinocembrin concentration is increased as
well. When the cultures were supplemented with 6 mM ATP, the strain exhibited the pinocembrin production of 23.00 mg/L.

Design and construction of the CRISPRi systems for repression of the genes
 acordo to previous research, the CRISPRi system can efficiently repress expression of targeted genes in E. coli, with no detectable off-target effects [20]. In the present study, we constructed our adjustable CRISPRi system in E. coli BL21 (DE3) and confirmed its effect by repressing the green fluorescent protein (gfp) expression. Three sgRNAs with different repression efficiency were designed and constructed. Three corresponding protospacer adjacent motif (PAM) was located on the 29th, 293th and 683th of gfp gene [19]. The closer the PAM region is to the initiation codon, the stronger the inhibition. As shown in the Additional file 1: Figure S1, compared with gfp-control, the fluorescence intensity of gfp-sgRNA-H, gfp-sgRNA-M and gfp-sgRNA-L could be decreased by 90.4%, 47.5% and 10.1% at 3 h, respectively. After 6 h, the fluorescence intensity of gfp-sgRNA-H, gfp-sgRNA-M and gfp-sgRNA-L could be decreased by 95.2%, 53.3% and 11.8% than that in gfp-control, respectively. These results indicated that CRISPRi system could be successfully repressing the expression of targeted protein in E. coli BL21 (DE3).

### Table 1 Plasmids used in this study

| Plasmid         | Plasmid description                                                                 | Source          |
|-----------------|--------------------------------------------------------------------------------------|-----------------|
| pACYC-dcas9     | P15A Replicon CmR                                                                    | Addgene         |
| pAC230-dcas9    | The dCas9 gene on plasmid pAC230-dCas9 was inserted into the BglII and XhoI restriction sites of the pACYCDuet-1 plasmid | This study      |
| pCDF303         | Trc promoter, CloE1 replicon, SmR                                                    | This study      |
| pCDF303-gfp-H   | Plasmid is used for high intensity repression of gfp                                 | This study      |
| pCDF303-gfp-M   | Plasmid is used for medium intensity repression of gfp                              | This study      |
| pCDF303-gfp-L   | Plasmid is used for low intensity repression of gfp                                  | This study      |
| pCDF-aroK-H     | Plasmid is used for high intensity repression of aroK                                | This study      |
| pCDF-aroK-M     | Plasmid is used for medium intensity repression of aroK                              | This study      |
| pCDF-aroK-L     | Plasmid is used for low intensity repression of aroK                                 | This study      |
| pCDF-metK-H     | Plasmid is used for high intensity repression of metK                                | This study      |
| pCDF-metK-M     | Plasmid is used for medium intensity repression of metK                              | This study      |
| pCDF-proB-H     | Plasmid is used for high intensity repression of proB                                 | This study      |
| pCDF-proB-M     | Plasmid is used for medium intensity repression of proB                              | This study      |
| pCDF-proB-L     | Plasmid is used for low intensity repression of proB                                 | This study      |
| pCDF-metK-H-proB-L | Plasmid is used for high intensity repression of metK and low intensity repression of proB | This study      |
| pCDF-metK-M-proB-L | Plasmid is used for medium intensity repression of metK and low intensity repression of proB | This study      |
| pCDF-metK-L-proB-L | Plasmid is used for low intensity repression of metK and low intensity repression of proB | This study      |
| pCDF-metK-H-proB-L | Plasmid is used for high intensity repression of metK and low intensity repression of proB | This study      |
| pCDF-metK-M-proB-L | Plasmid is used for medium intensity repression of metK and low intensity repression of proB | This study      |
| pCDF-metK-L-proB-L | Plasmid is used for low intensity repression of metK and low intensity repression of proB | This study      |
| pCDF-metK-H-proB-L | Plasmid is used for high intensity repression of metK and low intensity repression of proB | This study      |
| pCDF-metK-M-proB-L | Plasmid is used for medium intensity repression of metK and low intensity repression of proB | This study      |
| pCDF-metK-L-proB-L | Plasmid is used for low intensity repression of metK and low intensity repression of proB | This study      |
| pCDF-fabF-H     | Plasmid is used for high intensity repression of fabF                                 | This study      |
| pCDF-fabB-H     | Plasmid is used for high intensity repression of fabF                                 | This study      |
| pCDF-fabB-L     | Plasmid is used for low intensity repression of fabB                                  | This study      |
| pCDF-fumC-M     | Plasmid is used for medium intensity repression of fumC                                | This study      |
| pCDF-sucC-M     | Plasmid is used for medium intensity repression of sucC                                | This study      |
| pCDF-adhE-L     | Plasmid is used for low intensity repression of adhE                                   | This study      |
| pCDF-fabF-H-fabB-L | Plasmid is used for high intensity repression of fabF and low intensity repression of fabB | This study      |
| pCDF-fumC-M-sucC-M | Plasmid is used for high intensity repression of fumC and medium intensity repression of sucC | This study      |
| pCDF-fabF-H-fabB-L-fumC-M-sucC-M | Plasmid is used for high intensity repression of fabF, low intensity repression of fabB, medium intensity repression of fumC and medium intensity repression of sucC | This study      |
| pCDF-metK-H-proB-L-fabF-H-fabB-L-fumC-M-sucC-M | Plasmid is used for high intensity repression of metK, low intensity repression of proB, high intensity repression of fabF, low intensity repression of fabB, medium intensity repression of fumC, medium intensity repression of sucC and low intensity repression of adhE | This study      |
| pCDF-putA-H     | Plasmid is used for high intensity repression of putA                                  | This study      |
| pCDF-putA-H-proB-L | Plasmid is used for high intensity repression of putA and low intensity repression of proB | This study      |
Screening of different genes for increasing ATP levels and enhancing pinocembrin production

In order to increase the ATP supply for pinocembrin production, we tried to suppress ATP consumption from several related pathways. The pinocembrin metabolic pathway was constructed from L-phenylalanine. Shikimate, as phenylalanine precursor, can also produce shikimate-3-phosphate through ATP-dependent reaction. So aroK associated with this step was selected to be repressed (Fig. 1). Furthermore, phosphoenolpyruvate as shikimate precursor can also generate other ATP consumption pathways such as SAM and glutamate productions. Methionine is converted to SAM by metK-mediated ATP-dependent reaction. It follows that metK can be the candidate for repression. On the other hand, proB, glmA and argB, all involved in the synthesis of ATP-dependent downstream products of glutamate were selected as candidates for repression [13]. The CRISPRi strategy was used to fine tune

| Strain      | Strain description                          | Source        |
|-------------|---------------------------------------------|---------------|
| Empty       | BL21 (DE3) with pTrc-BOPAL-PA4CL, pRSF-CHS (Met) -CHI plasmid | This study    |
| Control     | Empty with pACYC-dCas9 plasmid              | This study    |
| gfp-sgRNA-H | Control with pCDF303-gfp-H plasmid          | This study    |
| gfp-sgRNA-M | Control with pCDF303-gfp-M plasmid          | This study    |
| gfp-sgRNA-L | Control with pCDF303-gfp-L plasmid          | This study    |
| Cri-aroK-H  | Control with pCDF-aroK-H plasmid            | This study    |
| Cri-glmA-H  | Control with pCDF-glmA-H plasmid            | This study    |
| Cri-argB-H  | Control with pCDF-argB-H plasmid            | This study    |
| Cri-metK-H  | Control with pCDF-metK-H plasmid            | This study    |
| Cri-metK-M  | Control with pCDF-metK-M plasmid            | This study    |
| Cri-metK-L  | Control with pCDF-metK-L plasmid            | This study    |
| Cri-proB-H  | Control with pCDF-proB-H plasmid            | This study    |
| Cri-proB-M  | Control with pCDF-proB-M plasmid            | This study    |
| Cri-proB-L  | Control with pCDF-proB-L plasmid            | This study    |
| Cri-metK-H-proB-L | Control with pCDF-metK-H-proB-L plasmid | This study    |
| Cri-metK-M-proB-L | Control with pCDF-metK-M-proB-L plasmid | This study    |
| Cri-metK-L-proB-L | Control with pCDF-metK-L-proB-L plasmid | This study    |
| Cri-fabF-H  | Control with pCDF-fabF-H plasmid            | This study    |
| Cri-fabB-L  | Control with pCDF-fabB-L plasmid            | This study    |
| Cri-fumC-M  | Control with pCDF-fumC-M plasmid            | This study    |
| Cri-sucC-M  | Control with pCDF-sucC-M plasmid            | This study    |
| Cri-adhE-L  | Control with pCDF-adhE-L plasmid            | This study    |
| Cri-fabF-H-fabB-L | Control with pCDF-fabF-H-fabB-L plasmid | This study    |
| Cri-fumC-M-sucC-M | Control with pCDF-fumC-M-sucC-M plasmid | This study    |
| Cri-A       | Control with pCDF-metK-H-proB-L plasmid     | This study    |
| Cri-M       | Control with pCDF-metK-H-proB-L-fumC-M-sucC-M plasmid | This study    |
| Cri-AM      | Control with pCDF-metK-H-proB-L-fabB-H-fabB-L-fumC-M-sucC-M plasmid | This study    |
| Cri-putA-H  | Control with pCDF-putA-H plasmid            | This study    |
| Cri-putA-H-proB-L | Control with pCDF-putA-H-proB-L plasmid | This study    |

**Fig. 2** The effect of ATP additive on pinocembrin production. Different concentrations of ATP ranging from 0 to 6 mM was added in the medium.
the intracellular ATP level to reach the requirements for pinocembrin biosynthesis. Five high-level repression strains, Cri-aroK-H, Cri-glnA-H, Cri-argB-H, Cri-proB-H and Cri-metK-H were constructed to improve the ATP supply (Fig. 3). As shown in the Fig. 3a, the intracellular ATP concentration of strains Cri-proB-H and Cri-metK-H was observed to be higher than the CK while the other stains Cri-aroK-H, Cri-glnA-H and Cri-argB-H showed less effective on the pinocembrin production. As shown in Fig. 3b, the strains Cri-proB-H and Cri-metK-H showed positive effect on pinocembrin production, which represented 18% and 104% increase compared with CK and showed a statistically significant (p < 0.05). The cinnamic acid that produced from the strains Cri-proB-H and Cri-metK-H displayed a 38% and 54% decrease than Control. Meanwhile, the strains Cri-aroK-H, Cri-glnA-H and Cri-argB-H led to a negative effect on pinocembrin production compared to Control.

**Optimization of repression efficiency of proB and metK**

To ascertain the effect of different repression level on pinocembrin production, different sgRNA was designed to bind the non-template DNA strand of the target gene at the initial, middle or terminal region. Therefore, corresponding PAM of proB was located on the 15th, 488th and 1076th of proB gene. Meanwhile, the PAM of metK was located on the 32th, 588th and 1115th of metK gene. As shown in Fig. 4a, an obvious increase in pinocembrin level (22.10, 25.49 and 27.18 mg/L) was observed in the strains Cri-proB-L, Cri-metK-M and Cri-metK-L while Cri-proB-M produced 16.65 mg/L pinocembrin, which is only 13% higher than that of the control strains. On the other hand, the ATP concentration of Cri-proB-H, Cri-proB-M, Cri-proB-L, Cri-metK-M and Cri-metK-L were increased by 20%, 26%, 40%, 27% and 38%, respectively (Fig. 4b). Particularly, with high-intensity inhibition of the metK gene, the ATP concentration could be increased by more than 45%. The order of conversion efficiency from high to low is Cri-metK-H, Cri-metK-M, Cri-metK-L, Cri-proB-L, Cri-proB-M and Cri-proB-H. Hence, the strains Cri-proB-L, Cri-metK-H, Cri-metK-M and Cri-metK-L were chosen as target genes for combination investigation. As shown in Fig. 4c, the strains Cri-metK-H-proB-L, Cri-metK-M-proB-L and Cri-metK-L-proB-L produced 40.59, 32.50 and 30.92 mg/L pinocembrin, respectively. The strains Cri-metK-H-proB-L was named as Cri-A for further investigation.

**Optimization of pinocembrin production by combining ATP and malonyl-CoA engineering strategy**

As the direct precursor of pinocembrin, malonyl-CoA was proved to be significant for the pinocembrin production level. Therefore, the intracellular ATP level was improved by high-level repression of proB and metK genes. As shown in Fig. 4a, an obvious increase in pinocembrin level (22.10, 25.49 and 27.18 mg/L) was observed in the strains Cri-proB-L, Cri-metK-M and Cri-metK-L while Cri-proB-M produced 16.65 mg/L pinocembrin, which is only 13% higher than that of the control strains. On the other hand, the ATP concentration of Cri-proB-H, Cri-proB-M, Cri-proB-L, Cri-metK-M and Cri-metK-L were increased by 20%, 26%, 40%, 27% and 38%, respectively (Fig. 4b). Particularly, with high-intensity inhibition of the metK gene, the ATP concentration could be increased by more than 45%. The order of conversion efficiency from high to low is Cri-metK-H, Cri-metK-M, Cri-metK-L, Cri-proB-L, Cri-proB-M and Cri-proB-H. Hence, the strains Cri-proB-L, Cri-metK-H, Cri-metK-M and Cri-metK-L were chosen as target genes for combination investigation. As shown in Fig. 4c, the strains Cri-metK-H-proB-L, Cri-metK-M-proB-L and Cri-metK-L-proB-L produced 40.59, 32.50 and 30.92 mg/L pinocembrin, respectively. The strains Cri-metK-H-proB-L was named as Cri-A for further investigation.
almost similar (Fig. 6b). As shown in Fig. 6c, pinocembrin production of Cri-AM was lower than that of the control strain (Fig. 6a), while the consumption of L-phenylalanine was decreased. The final OD600 of fabF was named as Cri-M for further investigation. M produced the highest pinocembrin titer (65.77 mg/L) than the strains Cri-L and Cri-H. The genes combination (addE and fabF) produce the higher pinocembrin production than the downstream genes combination (fumC and sucC) (supplementation of TCA intermediates) [21].

To increase the malonyl-CoA level, the effect of single genetic perturbations on pinocembrin production was investigated. As shown in Fig. 5a, medium repressing efficiency of sucC and fumC decreased the final OD600 by 13.5% and 7.3%. Low repressing efficiency of fabB and addE decreased the final OD600 by 17.4% and 23.1%. High repressing efficiency of fabF decreased the final OD600 by 9.4%. Meanwhile, the strains Cri-fabF-L, Cri-fabB-L, Cri-fumC-M, Cri-sucC-M produced an obvious increase in pinocembrin concentration (by over 50%). The strains Cri-addE-L increased the final pinocembrin production to 18.19 mg/L (Fig. 5b). It was also found that the upstream genes combination (fumC-M-sucC-M) and downstream genes combination (fabF-H-fabB-L) could produce the higher pinocembrin production than the single gene. However, the genes combination (fabF-H-fabB-L-addE-L) decreased the pinocembrin production than the strains Cri-fabF-H-fabB-L (data not shown). In addition, the strains Cri-fabF-H-fabB-L-fumC-M-sucC-M produced the highest pinocembrin titer (65.77 mg/L) (Fig. 5c). The strains Cri-fabF-H-fabB-L-fumC-M-sucC-M was named as Cri-M for further investigation.

In order to further increase the pinocembrin production, the ATP engineering strategy was combined with malonyl-CoA strategy to construct the integrated strain Cri-AM. The results showed that the OD600 of Cri-A and Cri-AM was lower than that of the control strain (Fig. 6a), while the consumption of L-phenylalanine was almost similar (Fig. 6b). As shown in Fig. 6c, pinocembrin production by strain Cri-AM (102.02 mg/L), was 68% and 155% higher than that of strain Cri-M and Cri-A. Further, the conversion rate of intermediate cinnamic acid by strains Cri-M and Cri-AM was higher than that of strains Cri-A and control (Fig. 6d). To confirm the effect of the CRISPRi system on the ATP and malonyl-CoA level, the intracellular ATP concentrations of Cri-A and Cri-AM were lower than that of the control strain at 0–36 h (Fig. 7b). Cri-A and Cri-M showed a higher ratio of ATP to ADP than that of the control (Fig. 7c). The strains Cri-AM and Cri-M produced a higher malonyl-CoA level, compared with the control and Cri-A from 0 to 48 h (Fig. 7d).

Optimization of cultivation and induction conditions

In order to further increase the pinocembrin production, the induction conditions and fermentation condition was optimized. First, strain Cri-AM was induced at OD600 values of 0.3, 0.6, 1.0, 1.5 and 2.0. As seen in Fig. 8a, the production of pinocembrin was maximized at an OD600 of 1.0 (110.54 mg/L). Meanwhile, five concentrations of IPTG, 0, 0.3, 0.6, 1.0 and 1.5 mM, were added into the culture when the OD600 of Cri-AM reached around 1.0. After 48 h incubation, the pinocembrin production exhibited the highest value of 110.44 mg/L, when the concentration of IPTG was 0.6 mM (Fig. 8b). In addition, the additive of L-phenylalanine concentration was optimized. The results showed that when the L-phenylalanine addition was 1.5 g/L, pinocembrin production reached the maximum (Fig. 8c). As shown in Fig. 8d, the pinocembrin production of Cri-AM reached 165.31 mg/L under the above optimized conditions.

Discussion

Biosynthesis of one molecule of pinocembrin requires one molecule of ATP in the pinocembrin metabolic pathway, thus, the intracellular ATP concentration might be significant for pinocembrin production. In this work, we identified a number of different genes-repression for increasing ATP and pinocembrin production in E. coli, and found that the repression of metK and proB based on CRISPRi system could enhance the pinocembrin production. The genes for the other enzyme tested, glnA, aroK and argB, had no effect. In addition, we optimized the gene repression level to increase the pinocembrin production and ATP level and further improved the pinocembrin production by multiple-gene repression of metK and proB. Combining optimization of culture, induction conditions and repression of the genes in the malonyl-CoA biosynthesis including fabF, fabB, fumC...
**Figure 1a**

Comparison of 
\( \text{OD}_{600} \) and Malonyl-CoA levels among different strains.

**Figure 1b**

Comparison of Pinocembrin and Cinnamic acid levels among different strains.

**Figure 1c**

Comparison of Pinocembrin and Cinnamic acid levels among different strains with a focus on specific strain configurations.
and sucC, a pinocembrin production of 11.2-fold higher than the Control were achieved. In the central metabolism of \textit{E. coli}, cofactors provide redox carriers for biosynthetic reactions, catabolic reactions and act as important agents in metabolism [22]. However, the low availability of cellular cofactor often impeded its utility for overproducing desired products. Although malonyl-CoA has been proved to be the limiting factor for flavonoids biosynthesis, the ATP availability in the synthetic pathway is also vital for pinocembrin production via ATP supplemented during the fermentation. In fact, the cell growth and maintenance, intracellular environment control, substrate transport and product export also require adequate supply of ATP [23]. In many cases, the optimization of the fermentation process to enhance the ATP level is an option [11]. ATP accumulation could also be elevated by gene knock-out strategies or small RNA regulation [13, 23, 24]. As CRISPRi system is advantageous over traditional gene-knockout strategies because of its ability to manipulate genes essential for growth [14], the development of CRISPRi system has offered an alternative approach to control the intracellular ATP level. The plasmid-based inducible CRISPRi system can regulate gene expression accurately at the transcriptional level in \textit{E. coli}. Here, depending on the pET28-\texttt{gfp} plasmid locus, the non-template of \texttt{gfp} combined with dCas9-sgRNA complex, which had been successfully repressed in \textit{E. coli} BL21(DE3) after induction by IPTG. It is found that CRISPRi system for ATP strategy did not generate growth repression in \textit{E. coli} (data not show).

Compared with Cri-\textit{proB-H} and Cri-\textit{proB-M}, Cri-\textit{proB-L} showed better effect on pinocembrin production. These finding indicate that the proline concentration

![Fig. 6 Time-course of OD600, substrate consumption and the products during 48 h fermentation by control strains, Cri-A, Cri-M and Cri-AM. a The effect of different strains on growth; b the effect of different strains on L-phenylalanine consumption; c the effect of different strains on pinocembrin production; d the effect of different strains on cinnamic acid production.](image-url)
might be important for *E. coli* metabolism and pinocembrin production. *MetK* was responsible for the final-product S-adenosylmethionine (SAM) in *E. coli*, while *proB* catalyze the reaction of the proline biosynthesis pathway and *putA* catalyze the reaction of the proline degradation pathway. Therefore, *Cri-putA-H* and *Cri-putA-H-proB-L* were constructed in order to detect whether the concentration of proline contribute to the improvement of pinocembrin production. As shown in Additional file 1: Figure S2, the strains *Cri-putA-H* decreased the pinocembrin production to 13.17 mg/L. The pinocembrin concentration in *Cri-putA-H-proB-L* was decreased by 30% when compared with that in *Cri-proB-L*. These results indicated that the further accumulation of proline concentration could not increase the ATP and pinocembrin production, and the repression of ATP consumption gene for proline or SAM biosynthesis was advantageous for pinocembrin production. In addition, we found that the combined strains *Cri-A* showed better effect than single gene interference. This demonstrated that coupling genetic modification to ATP strategy is benefit to identify the most suitable interventions. Further, the recombinant strains *Cri-A* and *Cri-AM* produced higher ATP, ATP/ADP ratio and pinocembrin production, compared with the control. These results indicate that the ATP concentration and the ATP/ADP ratio are associated with the pinocembrin production, and high level of ATP was benefit for pinocembrin biosynthesis. Moreover, the metabolism of malonyl-CoA, as with ATP, is highly connected with the metabolic network in *E. coli*. Due to its direct association with cell growth and synthesis of phospholipids and fatty acids, the intracellular availability of malonyl-CoA is limited [21]. Compared with ATP level, more malonyl-CoA was necessary for strains to achieve
a high yield of pinocembrin. By coupling malonyl-CoA genetic modification to ATP strategy, it was found that, the combined effects obviously improved the malonyl-CoA level and pinocembrin production from 0 to 48 h. These results indicate that the malonyl-CoA concentration is associated with the pinocembrin production. The present work demonstrates a way to approach the efficient biosynthesis of pinocembrin via ATP level strengthen and induction conditions optimization in E. coli.

**Conclusions**

In this work, we found that the addition of ATP contributes to the synthesis of pinocembrin. Five ATP-related genes were screened using the CRISPRi system, and inhibition of proB and metK was found to contribute to the accumulation of ATP and pinocembrin. On this basis, the repression intensity of proB and metK were optimized, and the results showed that low intensity repression of proB or high intensity repression of metK could better increase the production of pinocembrin. The effects of low intensity repression of proB and high intensity repression of metK on the synthesis of pinocembrin were investigated. The results of the study showed that the recombinant strain Cri-A produced a higher yield of pinocembrin (40.59 mg/L). In addition, ATP strategy coupled with the malonyl-CoA engineering and optimization of culture and induction condition, the production of pinocembrin by the recombinant strain increased by more than 7 times (102.02 mg/L) compared to the control.

**Additional file**

**Additional file 1:** Table S1. Primers and plasmids for single gene repression. Table S2. Primers and plasmids for repression of genes involved in malonyl-CoA consumption. Figure S1. The effect of different sgRNA on fluorescein on 3h and 6h. Figure S2. The effect of puzA on pinocembrin production.
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All authors read and approved the final manuscript.

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The authors declare that they have no competing interests.

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