Engineering *Escherichia coli* towards the production of prenylflavonoids

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Background

Prenylflavonoids are flavonoid-derived compounds that are characterized by the presence of a lipophilic prenylated side-chain in the flavonoid skeleton. These compounds have several recognized biological activities, namely estrogenic, antioxidant, anti-inflammatory and anticancer [1]. Xanthohumol and 8-prenylnaringenin, which are isolated from the *Humulus lupulus* plant, are prenylflavonoids with potential to be used in the treatment of several types of cancer and menopausal symptoms, respectively [2,3]. However, these compounds are only found in residual amounts in nature and their extraction from plants is difficult, expensive and renders impure products. Additionally, they are also hard to chemically synthesize. In addition, these processes are considered environmentally unfriendly [1]. For these reasons, the biosynthetic pathway reconstruction in a heterologous microorganism has emerged as an alternative to produce these compounds in higher amounts in a rapid, cheap, and more environmentally friendly way [4,5]. To produce prenylflavonoids in a microorganism, specifically xanthohumol and 8-prenylnaringenin, several genes must be expressed. In a first step, tyrosine can be converted into coumaric acid by tyrosine ammonia-lyase (TAL). Afterwards, coumaric acid is converted into coumaroyl-CoA by 4-coumarate-CoA ligase (4CL). Coumaroyl-CoA is further converted into naringenin chalcone by chalcone synthase (CHS). Afterwards, prenyltransferase (PT) converts naringenin chalcone into desmethylxanthohumol, which is converted into xanthohumol by desmethylxanthohumol 6'-*O*-methyltransferase (OMT1). Moreover, chalcone isomerase (CHI) converts naringenin chalcone into naringenin, which is converted into 8-prenylnaringenin by PT. Herein, we intend to design, construct, and validate a novel biosynthetic pathway to produce prenylflavonoids in *Escherichia coli* from simple carbon sources.

Methods

To construct the biosynthetic pathway to produce xanthohumol and 8-prenylnaringenin, the first steps of the biosynthetic pathway needed to be validated. To construct an engineered *E. coli* strain able to produce naringenin chalcone, TAL, 4CL and CHS genes from different organisms were selected. As a starting point, TAL from *Rhodotorula glutanis* (*Rg*TAL) and TAL from *Flavobacterium johnsoniae* (*Fj*TAL) were cloned into the pRSFduet-1 vector and were further expressed in three different *E. coli* strains (*E. coli* BL21, *E. coli* K12 MG1655 and *E. coli* M-PAR-121) to select the best enzyme and strain to produce coumaric acid. After selecting the best enzyme and strain combination to produce coumaric acid, 4CL and CHS steps were constructed and validated. Specifically, 4CL from *Arabidopsis thaliana* (*At*4CL), 4CL from *Vitis Vinifera* (*Vv*4CL), and 4CL from *Petroselinum crispum* (*Pc*4CL) were cloned into the

pACYCduet-1 vector. Moreover, CHS from *A. thaliana* (*At*CHS), CHS from *Petunia hybrida* (*Ph*CHS), and CHS from *Curcubita maxima* (*Cm*CHS) were cloned into pCDFduet-1 vector. All the fragments were cloned in the respective vector using restriction cloning. Different combinations of the 4CL and CHS genes were expressed in the best strain able to produce coumaric acid.

To evaluate the production of the desired compounds, *E. coli* strains carrying the different plasmids were cultured in 50 mL of LB in 250 mL flasks. The cultures were grown at 37˚C and 200 rpm until reaching an OD_{600nm} of 0.9. Afterwards, the protein expression was induced by adding IPTG (0.1 mM) and the cultures were maintained at 26˚C for 5 h. Subsequently, the cells were harvested by centrifugation and suspended in 50 mL of M9 medium and incubated at 26˚C for 63 h. Glucose (40 g/L) and IPTG (0.1 mM) were added at time 0 of induction. The experiments were conducted in triplicate.

Results

To choose the best enzymes and strain combination to produce coumaric acid, *Rg*TAL and *Fj*TAL were expressed in *E. coli* M-PAR-121*E. coli* K12 MG1655 and *E. coli* BL21. The production of coumaric acid from glucose for each case is illustrated in Figure 1.

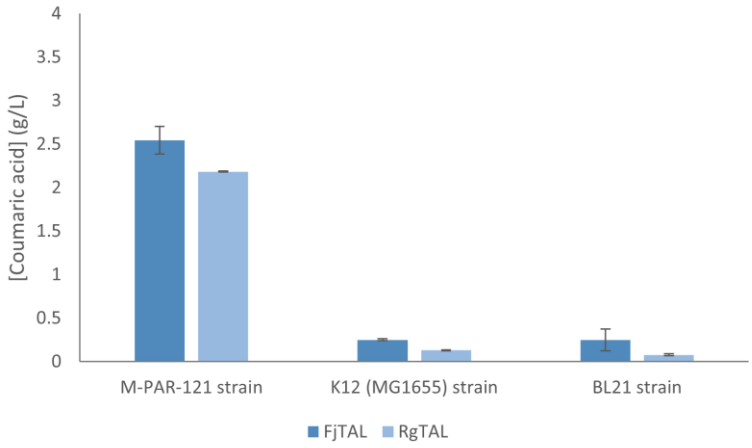

Figure 1. Coumaric acid production in three *Escherichia coli* strains (*E. coli* BL21, *E. coli* K12 MG1655 and *E. coli* M-PAR-121) expressing tyrosine ammonia-lyase (TAL) from *Rhodotorula glutanis* (*Rg*TAL) or TAL from *Flavobacterium johnsoniae* (*Fj*TAL). The values presented correspond to the average of three independent tests ± standard deviation.

Comparing the *E. coli* strains, the highest production of coumaric acid was obtained when the *E. coli* M-PAR-121 strain expressed either *Fj*TAL or *Rg*TAL. However, in all the strains, the production of coumaric acid was higher when *Fj*TAL was expressed. The highest coumaric acid production was obtained in the *E. coli* M-PAR-121 strain expressing *Fj*TAL (2.54 g/L of coumaric acid). This occurs since the *E. coli* M-PAR-121 is able to produce high amounts of tyrosine from glucose since it is a tyrosine-overproducing strain. For this reason, higher amounts of tyrosine are available to be converted into coumaric acid [6]. Thus, this strain and enzyme were chosen to be used

in the construction of the prenylflavonoids biosynthetic pathway.

After selecting the *E. coli* M-PAR-121 strain expressing *Fj*TAL enzyme as the best coumaric acid producer, strains containing different biosynthetic pathways were constructed by combining three 4CL genes (*At*4CL, *Vv*4CL, and *Pc*4CL) and three CHS genes (*At*CHS, *Ph*CHS, and *Cm*CHS). The production of naringenin chalcone and coumaric acid from glucose were evaluated in the nine constructed strains (**Figure 2**).

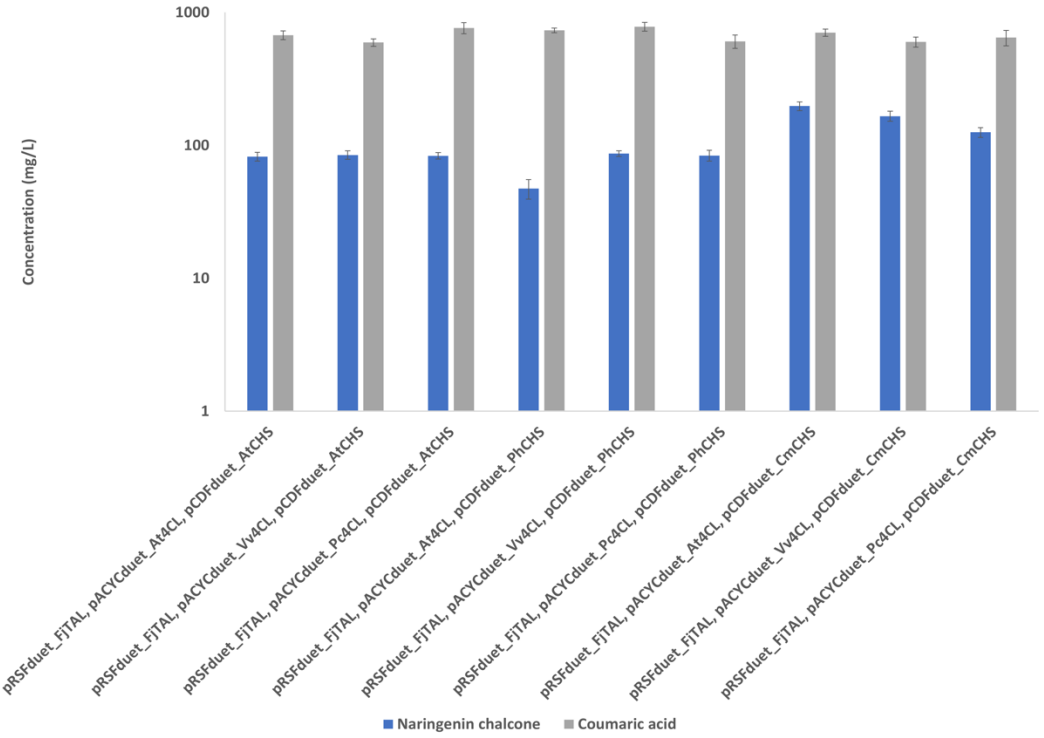

Figure 2. Naringenin chalcone and coumaric acid production in the constructed *E. coli* M-PAR-121 strains expressing tyrosine ammonia-lyase (TAL) from *Flavobacterium johnsoniae* (*Fj*TAL) in combination with three 4-coumarate-CoA ligase (4CL) genes (4CL from *Arabidopsis thaliana* (*At*4CL), 4CL from *Vitis Vinifera* (*Vv*4CL), and 4CL from *Petroselinum crispum* (*Pc*4CL)) and three chalcone synthase (CHS) genes (CHS from *A. thaliana* (*At*CHS), CHS from *Petunia hybrida* (*Ph*CHS), and CHS from *Curcubita maxima* (*Cm*CHS)). The values presented correspond to the average of three independent tests ± standard deviation.

As can be observed in **Figure 2**, the highest production of naringenin chalcone was achieved with the pathways holding the *Cm*CHS gene. This demonstrates the higher efficiency of this gene to convert coumaroyl-CoA into naringenin chalcone. The highest production of naringenin chalcone (197.9 mg/L) was obtained in the *E. coli* M-PAR-121 strain expressing *Fj*TAL, *At*4CL and *Cm*CHS. These results of naringenin chalcone production are not easily comparable to the literature since it is only reported naringenin production that is produced from naringenin chalcone by CHI. Comparing the titers of naringenin obtained in flask fermentations using glucose as substrate reported in the literature, the concentration of naringenin chalcone achieved in this study was higher, which is very promising [7]. Nevertheless, we still need to include CHI in our pathway and it is known that the titers usually decrease as the number of steps of the pathway increase. Moreover, it was also possible to observe that high amounts of coumaric acid were accumulated during the production experiments and it was not fully converted into naringenin chalcone. This demonstrates that 4CL is a limiting step and the

discovery of more efficient 4CLs is of upmost importance for the success this pathway construction.

Conclusion

In this study, it was possible to select the best strain and enzyme combination to produce coumaric acid. The highest coumaric acid production (2.54 g/L) was obtained in the *E. coli* M-PAR-121 strain expressing *Fj*TAL. Afterwards, this strain was used as platform to select the best pathway to produce naringenin chalcone. The best naringenin chalcone production was obtained in the *E. coli* M-PAR-121 strain expressing *Fj*TAL, *At*4CL and *Cm*CHS (197.9 mg/L). Therefore, this constructed strain will be used as platform to produce prenylflavonoids.

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