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Hydroxycinnamic acids and curcumin production in engineered *Escherichia coli* using heat shock promoters

Joana L. Rodrigues[a,c,d,*], Márcia R. Couto[a], Rafael G. Araújo[a,1], Kristala L.J. Prather[b,c,d], Leon Kluskens[a,2], Lígia R. Rodrigues[a,c,d,*]

[a] Centre of Biological Engineering, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal
[b] Department of Chemical Engineering, Synthetic Biology Engineering Research Center (SynBERC) Massachusetts Institute of Technology, Cambridge, MA 02139, USA
[c] MIT-Portugal Program, Cambridge, MA, USA
[d] MIT-Portugal Program, Lisbon, Portugal

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ABSTRACT

Hydroxycinnamic acids and curcumin are compounds with great therapeutic potential, including anti-cancer properties. In this study, *p*-coumaric acid, caffeic acid and curcumin were produced in *Escherichia coli*. Their production was induced by heat using the *dnaK* and *ibpA* heat shock promoters. The ribosome binding site (RBS) used was tested and further optimized for each gene to assure an efficient translation. *p*-Coumaric acid was successfully produced from tyrosine and caffeic acid was produced either from tyrosine or *p*-coumaric acid using tyrosine ammonia lyase (TAL) from *Rhodotorula glutinis*, 4-coumarate 3-hydroxylase (C3H) from *Saccharothrix espanaensis* or cytochrome P450 CYP199A2 from *Rhodopseudomonas palustris*. The highest *p*-coumaric acid production obtained was 2.5 mM; caffeic acid production reached 370 μM. Regarding curcumin, 17 μM was produced using 4-coumarate-CoA ligase (4CL1) from *Arabidopsis thaliana*, diketide-CoA synthase (DCS) and curcumin synthase 1 (CURS1) from *Curcuma longa*. Stronger RBSs and/or different induction conditions should be further evaluated to optimize those production levels. Herein it was demonstrated that the biosynthetic pathway of *p*-coumaric acid, caffeic acid and curcumin in *E. coli* can be triggered by using heat shock promoters, suggesting its potential for the development of new industrial bioprocesses or even new bacterial therapies.

1. Introduction

Hydroxycinnamic acids such as *p*-coumaric and caffeic acids are phenylpropanoids naturally synthesized by plants from amino acids. They can be found in several fruits and vegetables, and have a well-known antioxidant activity. In addition, they have been reported to have other benefits including anti-inflammatory and anticancer activities [1,2]. Due to its natural radical scavenging properties, studies showed that *p*-coumaric acid protects the rat heart against the oxidative stress caused by the anticancer drug doxorubicin and that the pre-treatment and co-administration of *p*-coumaric acid can be highly beneficial [2,3]. In addition to several reports showing its anticancer and anti-apoptosis properties [1], caffeic acid has also been shown to possess antiviral and antidiabetic activities [4–6]. All of these beneficial properties encourage the application of *p*-coumaric and caffeic acids in pharmaceuticals and healthy or functional foods. In addition, these hydroxycinnamic acids are precursors to an enormous array of secondary metabolites with desirable and relevant properties, such as curcumin. Curcumin is a polyphenolic compound that has been used as a food additive, as well as in traditional medicine [7] due to its several therapeutic properties including anticancer, antioxidant, anti-inflammatory, anti-HIV, anti-Alzeimer's and anti-Parkinson [8–11]. All of these compounds are accumulated at low levels in plants and their extraction is complex, low, inefficient, environ-

Abbreviations: 4CL, 4-coumarate-CoA ligase; C3H, 4-coumarate 3-hydroxylase; CURS1, curcumin synthase 1; DCS, diketide-CoA synthase; GFP, green fluorescence protein; IPTG, isopropyl β-D-1-thiogalactopyranoside; OD, optical density; RBS, ribosome binding site; TAL, tyrosine ammonia lyase; TIR, translation initiation rate; UHPLC, ultra-high-performance liquid chromatography.

* Corresponding authors at: Centre of Biological Engineering, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal.
E-mail addresses: joana.joanalucia@deb.uminho.pt (J.L. Rodrigues), marciacouto93@gmail.com (M.R. Couto), rafa.gomes.ar@gmail.com (R.G. Araújo), kljp@mit.edu (K.L.J. Prather), lrmr@deb.uminho.pt (L.R. Rodrigues).
[1] Biorefinery Group, Food Research Department, School of Chemistry, Autonomous University of Coahuila, 25280 Saltillo, Coahuila, Mexico.
[2] Deceased on April 1st 2016.

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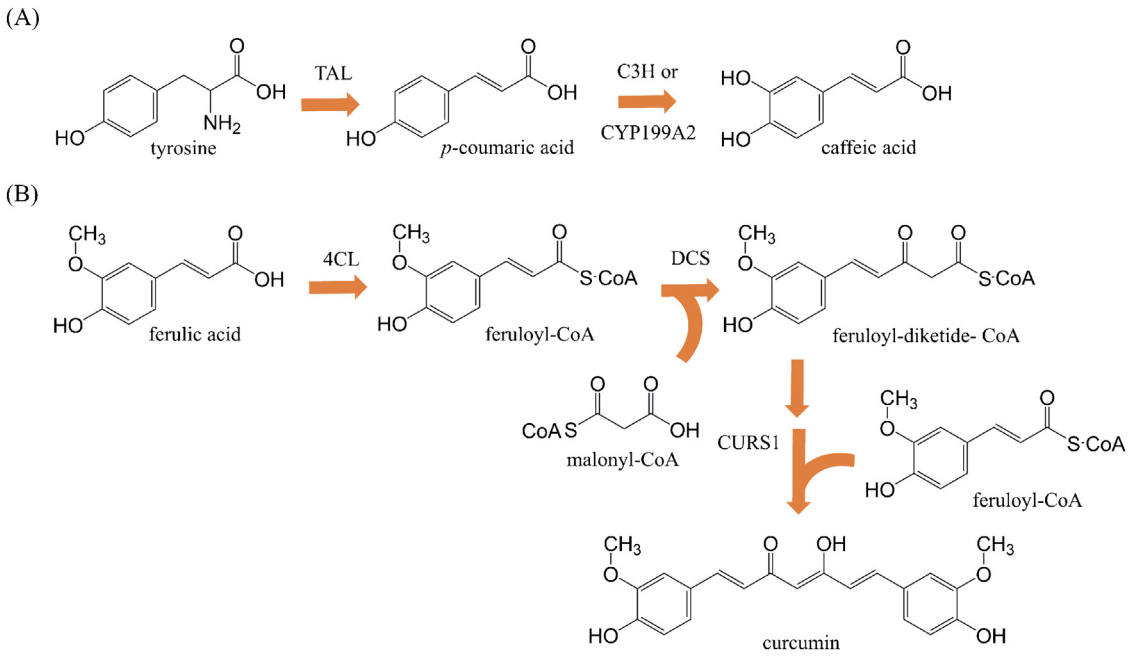

Fig. 1. *p*-Coumaric acid, caffeic acid (A) and curcumin biosynthetic pathway (B). TAL: Tyrosine ammonia lyase; C3H: 4-coumarate 3-hydroxylase; 4CL: 4-coumarate-CoA ligase; DCS: diketide-CoA synthase; CURS1: curcumin synthase 1.

mentally unfriendly and expensive [12]. In addition, their isolation as pure compounds remains inefficient and their availability is limited by regional variations and seasonality [13]. Also, their chemical synthesis is very difficult. Therefore, microbial conversion comprises a promising alternative for the production of hydroxycinnamic acids and curcumin. *p*-Coumaric acid, caffeic acid and curcumin were recently produced in *Escherichia coli* using different artificial biosynthetic pathways [14–19]. Previously, we reported the production of hydroxycinnamic acids and curcumin in *E. coli* using the biosynthetic pathway illustrated in Fig. 1 [17,18].

E. coli has been the host of choice for the expression of recombinant proteins given its ability to produce high quantities at low costs. However, in large-scale productions, chemical inducers, such as isopropyl β-D-1-thiogalactopyranoside (IPTG), can be expensive and toxic [20,21] and their presence in waste effluents or in the final product must be eliminated, especially in the production of pharmaceutical-grade proteins and other products intended for human use [22]. Constitutive promoters or promoters induced by starvation of an essential nutrient, or by shift in a physical or physicochemical factor, such as temperature or pH, allow an inducer-free environment for heterologous protein expression [23]. Thermal induction in *E. coli* is carried out by increasing the temperature (usually 37 °C) to 42 °C or higher for a certain period and then, shifting it down [24–31]. A thermal induction strategy has the potential of reducing the fermentation cost since expensive chemicals or special media are not required. In addition, it simplifies the downstream processing since culture handling and contamination risks are minimized [24,32]. All of these aspects are very important when producing therapeutic recombinant proteins and products at an industrial scale [24]. Induction by heat can also be very advantageous in therapeutic approaches, for example in bacterial therapies. These therapies can be combined with laser or ultrasound treatments and the temperature increase would trigger the production in situ of the desired compounds, such as hydroxycinnamic acids and curcumin.

Due to the need of finding and characterizing new parts to use in synthetic biology approaches and the advantages of using promoters not chemically induced, we previously studied *E. coli* heat shock promoters [33]. The *dnaK* and *ibpA* heat shock promoters were used with a synthetic ribosome binding site (RBS) and the green fluorescence protein (GFP) to design and construct stress probes, which were further used to evaluate the promoter strength and their potential use in synthetic biology applications [33]. In the current study, the heat shock induction system was coupled to the artificial biosynthetic pathways leading to the production of *p*-coumaric acid, caffeic acid and curcumin. The *dnaK* and *ibpA* promoters and different synthetic RBSs with several strengths were used. The results gathered herein demonstrate that *p*-coumaric acid, caffeic acid and curcumin can be produced in *E. coli* using heat shock promoters and that synthetic biology tools can help to improve their production.

2. Materials and methods

2.1. Bacterial strains, plasmids and chemicals

E. coli NZY5α competent cells (NZYTech, Lisbon, Portugal) were used for molecular cloning and vector propagation and *E. coli* K-12 MG1655(DE3) [34] was used as host. *E. coli* K-12 ER2925 (NEB, Ipswich, MA, USA) competent cells were used whenever restriction endonucleases sensitive to *E. coli* K-12 methylation patterns were required. Table 1 summarizes the characteristics of all strains and plasmids used. Synthesis and amplification of TAL, C3H, CYP199A2, Pdr, Pux, 4CL1, DCS and CURS1 was previously described [17,18]. The DNA sequences of the codon-optimized genes are provided in Table S1. pAC-4CL1 plasmid was kindly provided by Claudia Schmidt-Dannert [35] (Addgene plasmid # 35947).

L-Tyrosine, *p*-coumaric and caffeic acid were purchased from Sigma-Aldrich (Steinheim, Germany); ferulic acid from Acros (Geel, Belgium); curcumin from Fisher Scientific (Loughborough, UK) and Luria-Bertani (LB) medium from NZYTech (Lisbon, Portugal). Glucose (Acros), Na_2HPO_4 (Scharlau, Sentmenat, Spain), $MgSO_4$, KH_2PO_4 (Riel-deHaën, Seelze, Germany), NH_4Cl, NaCl, $CaCO_3$ (Panreac, Barcelona, Spain) and thiamine (Fisher Scientific, Loughborough, UK) were used to prepare the M9 modified salt medium. The following mineral traces and vitamins were