Synthetic biology strategies for the production of plant polyphenolic compounds

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Background

Polyphenols are secondary metabolites naturally produced in plants with an estimated market size of USD 2.26 billion by 2027[1]. These compounds have several biological and potential therapeutic activities such as anti-inflammatory, antioxidant and anticancer. However, polyphenols are extracted from plants where they accumulate in low amounts over long growth periods. Moreover, a high investment of water, land and time is required, and pests and extreme weather cause insecurity in the supply chain. In addition, the extraction process is difficult, inefficient, expensive and environmentally unfriendly since it requires the separation of other compounds with similar chemical structures. Therefore, in the past decade, several efforts have been made to produce polyphenols using synthetic biology approaches[2–9]. The use of microbial chassis to produce polyphenols using combinatorial biosynthesis has several benefits as it is not limited by plant availability or environmental factors and it is a renewable, environmentally friendly and sustainable approach. Herein, we report the construction of artificial pathways for the production of curcuminoids and furanocoumarins using *Escherichia coli* as chassis. These compounds can be produced from the amino acid tyrosine or hydroxycinnamic acids (coumaric acid, ferulic acid) as precursors and have in common the phenylpropanoids pathway.

Methods

Curcuminoids pathway involves 6 enzymes: tyrosine ammonia lyase (TAL), 4-coumarate 3-hydroxylase (C3H), caffeic acid *O*-methyltransferase (COMT), 4-coumarate-CoA ligase (4CL), diketide-CoA synthase (DCS), and curcumin synthase (CURS1) (Figure 1). Curcuminoids pathway was divided in two modules, the first module included TAL, C3H and COMT and the second one 4CL, DCS and CURS1 and they were optimized separately. The genes were cloned in the Duet plasmids from Novagen. In a first approach, each gene of the module was cloned in a single plasmid. However, to reduce the metabolic burden, the three genes of each module were distributed in two plasmids. After optimizing both modules by testing different induction approaches and plasmids, curcumin and other curcuminoids were produced from tyrosine (using the whole pathway) in mono-culture. Subsequently, the potential of a co-culture approach was evaluated to further improve curcuminoids production by reducing cells metabolic burden. We used one *E. coli* strain able to convert tyrosine to ferulic acid and another able to convert the hydroxycinnamic acids produced by the first one to curcuminoids. To follow the co-culture population using the blue-white

screening method, we used CRISPR-Cas9 tool to disrupt the β-galactosidase gene in one of the strains.

Regarding furanocoumarins pathway, the first steps are the same as the ones in the curcuminoids pathway with TAL and 4CL enzymes that convert tyrosine to coumaroyl-CoA. Then coumaroyl-CoA 2'-hydroxylase (C2'H) can be used to produce several coumarins (Figure 1). The expression of prenyltransferase (PT) and psoralen synthase (PS) enzymes from furanocoumarins pathway was also studied and engineered.

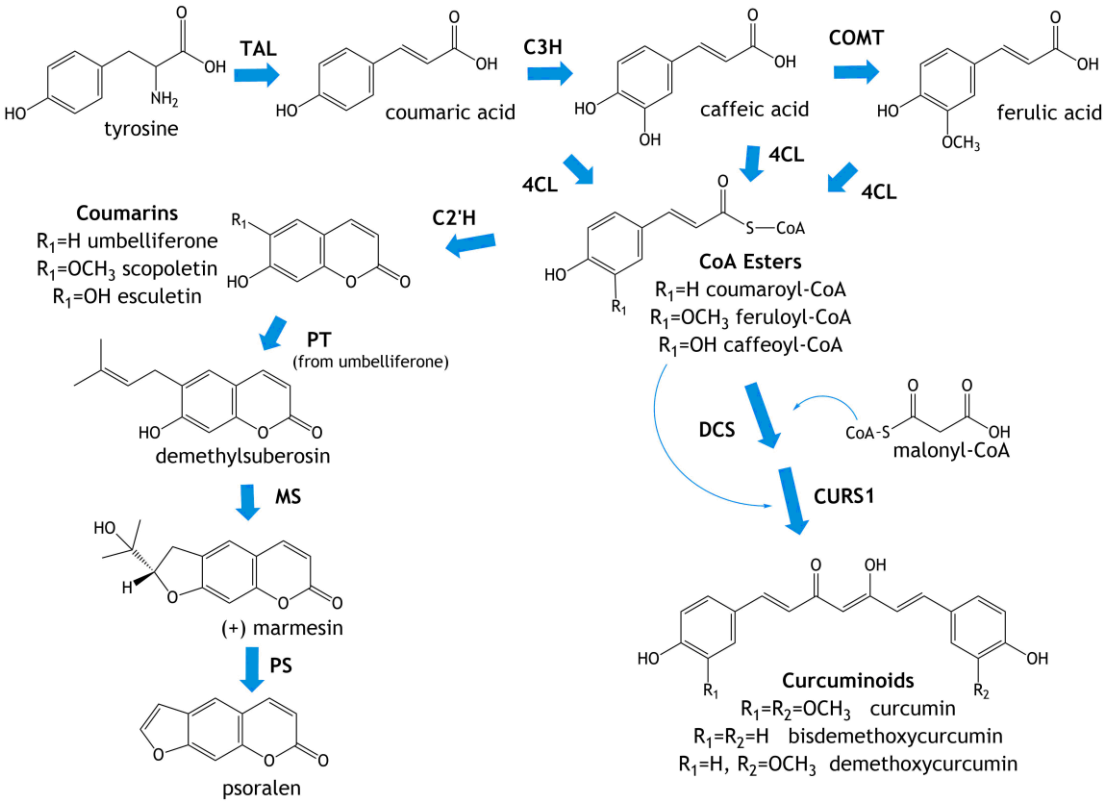

Figure 1: Biosynthetic pathway for curcuminoids and furanocoumarins production. 4CL, 4-coumarate-CoA ligase; C2'H, coumaroyl-CoA 2'-hydroxylase; C3H, 4-coumarate 3-hydroxylase; COMT, caffeic acid *O*-methyltransferase; CURS1, curcumin synthase 1; DCS, diketide-CoA synthase; MS, marmesin synthase; PS, psoralen synthase; PT, prenyltransferase; TAL, tyrosine ammonia lyase.

Results

In this study, we did a step-by-step optimization of the curcuminoids biosynthetic pathway. Firstly, we divided the pathway in two modules. The first module contained the genes of the pathway that converted tyrosine to ferulic acid and the second one contained the genes that converted the hydroxycinnamic acids to curcuminoids. The optimization of the two modules of the pathway led to the production of 1325.1 µM of ferulic acid and 1529.5 µM of pure curcumin, the highest concentrations ever reported (Figure 2a and Figure 2b, respectively). Curcumin was obtained from ferulic acid with a percent yield of 100%. Afterwards, the biosynthetic pathway was assembled and curcumin and other curcuminoids were produced from tyrosine in mono-culture

(Figure 3a). Using this strategy, the curcumin (16.6 µM) and total curcuminoids (19 µM) production (Figure 2c) increased compared to previous studies[7,8].

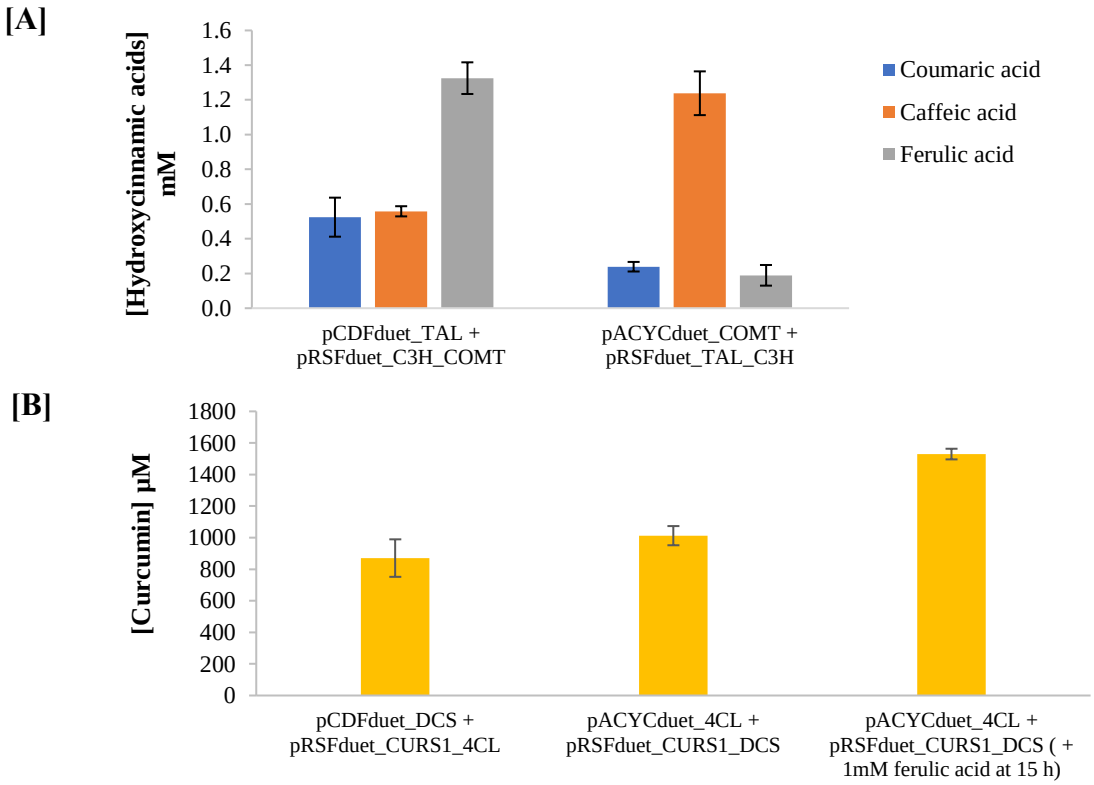

Figure 2: Hydroxycinnamic acids and curcuminoids production by *E. coli*. (A) Hydroxycinnamic acids production by *E. coli* from tyrosine and carrying pCDFduet_TAL and pRSFduet_C3H_COMT or pRSFduet_TAL_C3H and pACYCduet_COMT. (B) Curcumin production by *E. coli* Δ*lacZ* from ferulic acid and carrying pCDFduet_DCS and pRSFduet_CURS1_4CL or pACYCduet_4CL and pRSFduet_CURS1_DCS. TAL, tyrosine ammonia lyase; C3H, 4-coumarate 3-hydroxylase; COMT, caffeic acid *O*-methyltransferase; 4CL, 4-coumarate-CoA ligase; DCS, diketide-CoA synthase; CURS1, curcumin synthase 1.

Since this production was still low, we tested a co-culture strategy that led to 6.6 times increase of total curcuminoids (125.8 µM) as compared to the mono-culture system (Figure 3b). The curcuminoids production from tyrosine using this co-culture approach corresponds to a 6817% enhancement[7,8]. Firstly, co-culture population was followed by the production of curcuminoids that conferred a yellow/orange color to one of the cultures. However, the colors were difficult to distinguish when low production was obtained. Therefore, CRISPR-Cas9 was used to disrupt *lacZ* in one of the strains and the colonies were screened using the blue-white screening method. Different co-culture inoculation ratios were tested and it was observed that when the strain carrying the first part of the pathway was inoculated in higher amount the production of curcuminoids was favored. Through the co-culture monitoring it was concluded that this strain carrying the first module of the pathway was at growth disadvantage since when both

strains were inoculated in the same proportion, at 63 h of growth the proportion was around 20%: 80%.

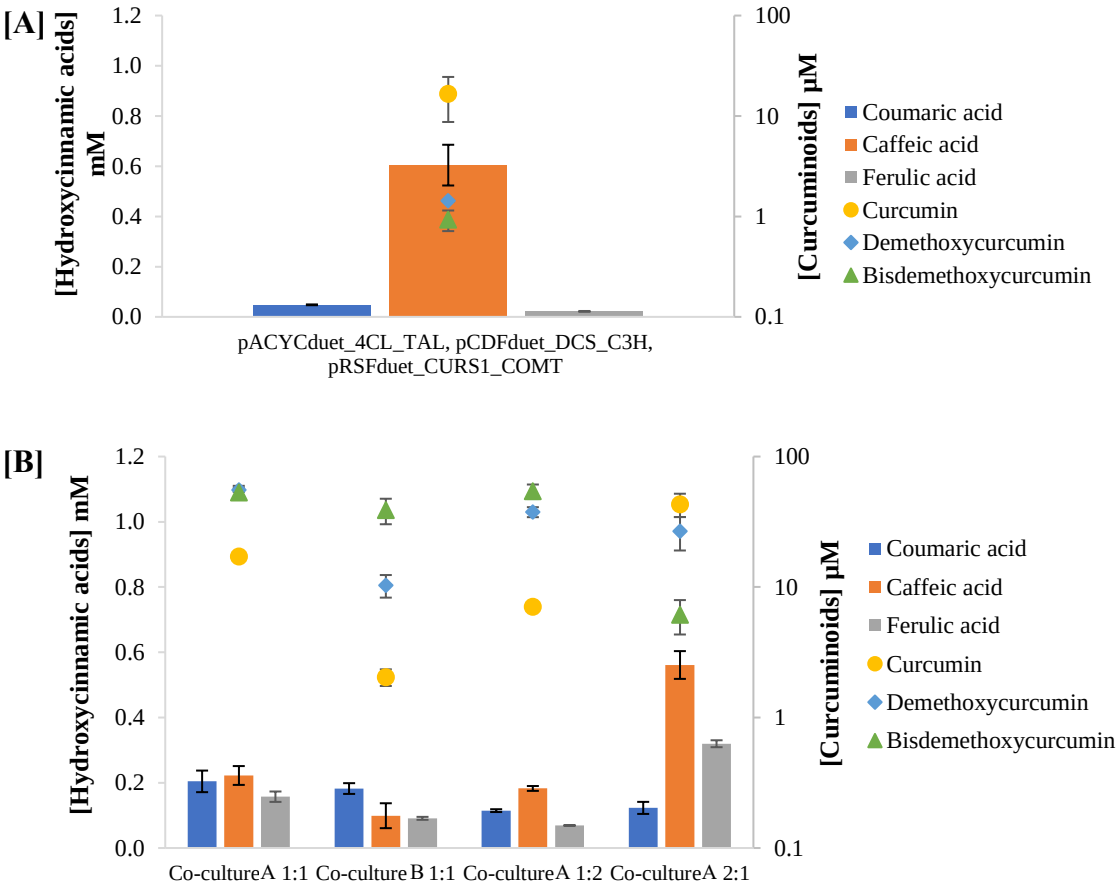

Figure 3: Curcuminoids production from tyrosine by *E. coli*. (A) Production of curcuminoids by *E. coli* mono-culture system from tyrosine and carrying pACYCduet_4CL_TAL, pCDFduet_DCS_C3H and pRSFduet_CURS1_COMT. (B) Production of curcuminoids by a co-culture system from tyrosine. In co-culture A, *E. coli* carried pCDFuet_TAL and pRSFduet_C3H_COMT and *E. coli* Δ*lacZ* carried pCDFduet_DCS and pRSFduet_CURS1_4CL. In co-culture B, *E. coli* carried pRSFduet_TAL_C3H and pACYCduet_COMT and *E. coli* BL21 (DE3) Δ*lacZ* carried pACYCduet_4CL and pRSFduet_CURS1_DCS. Co-culture A was tested using different inoculation ratios (1:1, 2:1 and 1:2). TAL, tyrosine ammonia lyase; C3H, 4-coumarate 3-hydroxylase; COMT, caffeic acid *O*-methyltransferase; 4CL, 4-coumarate-CoA ligase; DCS, diketide-CoA synthase; CURS1, curcumin synthase 1.

Based in the curcuminoids pathway, a similar pathway was constructed to produce coumarins. The enzymes 4CL and C2'H were used to produce the coumarins umbelliferone, scopoletin and esculetin from coumaric acid, ferulic acid and caffeic acid, respectively. Glutathione S- transferase tag was used to assist the proper folding of C2'H from *Ipomoea batatas*. However, most of the protein remained as inclusion bodies. Approximately 100-263 µM of each coumarin was produced. Umbelliferone,

precursor of furanocoumarins, was the coumarin produced in higher amount (Figure 4). Umbelliferone production from tyrosine was also obtained (126 µM) by adding TAL enzyme to the pathway. More recently, PT and PS protein were engineered in order to remove N-terminal sequences correspondent to transit peptides related to plastid localization. The sequences were replaced by other sequences known to increase protein expression by increasing solubility. Co-expression of chaperones (GroESL system) to facilitate furanocoumarins folding is also being tested.

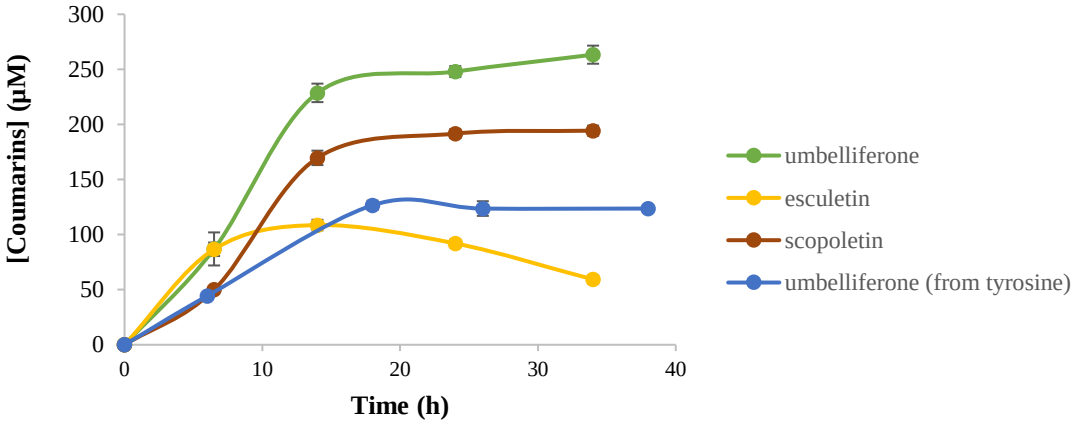

Figure 4: Coumarins production in *E. coli* from hydroxycinnamic acids and tyrosine.

Conclusion

To our knowledge, the curcuminoids productions herein obtained correspond to the highest titers ever reported. The enormous potential of synthetic biology and modular co-culture engineering to produce curcuminoids from tyrosine or other compounds that require several enzymatic steps was herein demonstrated. The results obtained comprise a significant step towards the large-scale production of these valuable compounds. Regarding the furanocoumarins heterologous production, it is still in an early stage and has a long way to go. Nevertheless, recent advancements on some of the enzymes of the pathway are encouraging.

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