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Potential of *Synechocystis* PCC 6803 as a novel cyanobacterial chassis for heterologous expression of enzymes in the *trans*-resveratrol biosynthetic pathway

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ABSTRACT

Selected model strains of phototrophic cyanobacteria have been genetically engineered for heterologous expression of numerous enzymes. In the present study, we initially explored the heterologous expression of enzymes involved in *trans*-resveratrol production, namely, the production of tyrosine ammonia-lyase, coumaroyl CoA-ligase, and stilbene synthase, in the unicellular cyanobacterium *Synechocystis* PCC 6803. Under the promoters P*trc*1Ocore and P*trc*1O, the respective genes were transcribed and translated into the corresponding soluble proteins at concentrations of 16–34 μg L^{-1}. The expression levels of these enzymes did not affect the growth rate of the cyanobacterial cells. Interestingly, coumaroyl CoA-ligase expression slightly increased the chlorophyll *a* content of the cells. Overall, our results suggest that the complete pathway of *trans*-resveratrol production can be engineered in *Synechocystis* PCC 6803.

1. Introduction

For decades, the phenylpropanoid metabolic pathway has been recognized as an important secondary metabolic pathway in plants. This pathway is involved in the production of numerous bioactive molecules that protect plants under adverse environmental conditions, such as UV radiation, photo-damage, insect attack and drought. *p*-coumaric acid is a crucial intermediate of flavonoids, coumarins, stilbenes and other secondary metabolites. It is generally produced by the action of phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) on a phenylalanine substrate, followed by cinnamic acid 4-hydroxylase (C4H; EC 1.14.13.11). The bacterial enzyme tyrosine ammonia-lyase (TAL; EC 4.3.1) also converts L-tyrosine to *p*-coumaric acid. The produced *p*-coumaric acid is then converted by coumaroyl CoA-ligase (4CL; EC 6.2.1.12) to *p*-coumaroyl Coenzyme A (CoA), a precursor of many bioactive compounds (Fig. 1) [1].

The stilbene compound 3, 5, 4′-*trans*-trihydroxystilbene (*trans*-resveratrol) has been studied for its anti-cancer, anti-viral, neuroprotective, anti-aging and anti-inflammatory effects [2–5]. To produce *trans*-resveratrol, *p*-coumaroyl CoA is condensed with three molecules of malonyl CoA in the presence of stilbene synthase (STS; EC 2.3.1.95). Previously, genes encoding different enzymes of the *trans*-resveratrol biosynthetic pathway have been extracted from various sources, such as *TAL* from *Rhodobacter sphaeroides*, *4CL* from tobacco (*Nicotiana tabacum*) and *Arabidopsis thaliana*, and *STS* from grapevine (*Vitis vinifera*), and introduced into yeast (*Saccharomyces cerevisiae*) and *Escherichia coli*. These organisms are the usual expression hosts for *trans*-resveratrol production [6–8]. A bacterial *TAL* has also been introduced into *A. thaliana*, increasing the metabolic flux into the phenylpropanoid pathway [9].

Abbreviations: C4H, cinnamic acid 4-hydroxylase; chl *a*, chlorophyll *a*; 4CL, coumaroyl CoA-ligase; PAL, phenylalanine ammonia-lyase; PBS, phosphate-buffered saline; PBST, PBS containing Tween 20; RT-PCR, reverse transcription polymerase chain reaction; RNAP, RNA polymerase; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; STS, stilbene synthase; TAL, tyrosine ammonia-lyase.

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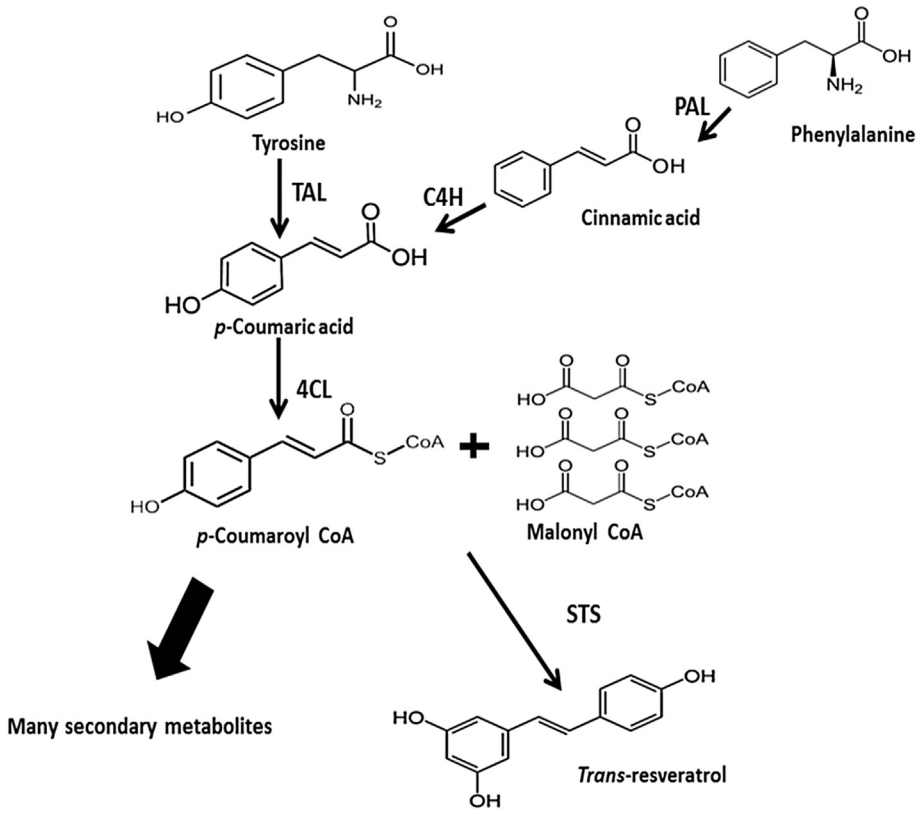

Fig. 1. The *trans*-resveratrol biosynthetic pathway. TAL: tyrosine ammonia-lyase, PAL: phenylalanine ammonia-lyase, C4H: cinnamic acid 4-hydroxylase, 4CL: coumaroyl CoA-ligase, and STS: stilbene synthase.

However, the heterologous expression of plant genes in microorganisms is limited by the different biomolecular systems of these two organisms; consequently, microorganisms cannot express large amounts of plant products. The expression of plant genes in plant cell cultures is more compatible, but the cultivation time is long. For large-scale production of bioactive molecules, cyanobacteria are attractive candidates because of the following: 1) they grow photoautotrophically, 2) advanced tools are now available for designing and genetically engineering selected model strains, and 3) cyanobacteria have proven ability to produce numerous non-native metabolites. The unicellular cyanobacterium *Synechocystis* PCC 6803 (hereon referred to as *Synechocystis*) has been widely exploited as a model cyanobacterial expression host. For instance, cyanobacteria containing the plant thioesterase gene produces a feasible yield of fatty acids [10]. Lindberg et al. (2010) optimized the isoprene synthase gene from the plant kudzu (*Pueraria montana*) for codon preference and expressed it in *Synechocystis*. The expression level was higher in the optimized gene than in the wild-type [11]. Since these findings were published, cyanobacteria have been extensively studied as a source of solar fuel production. Plant terpenoids may also be heterologously produced in cyanobacteria [12]. Recently, the gene encoding TAL from *Saccharothrix espanaensis* was successfully expressed in *Synechocystis* and *p*-coumaric acid was detected [13]. To our knowledge, we present the first successful expression of genes encoding three enzymes of the *trans*-resveratrol biosynthetic pathway in *Synechocystis*. Our findings are a prerequisite for engineering the complete pathway in cyanobacteria.

2. Materials and methods

2.1. Escherichia coli strains and growth condition

E. coli strain DH5αZ1 (Thermo Fisher Scientific, Stockholm, Sweden) was used for DNA cloning and plasmid construction. *E. coli* strain HB101 (Thermo Fisher Scientific, Stockholm, Sweden) was used for introducing the genes into *Synechocystis* by triparental mating. *E. coli* strains were cultured at 37 °C with shaking at 250 rpm in LB medium containing ampicillin (Sigma-Aldrich Sweden AB, Stockholm, Sweden) at 100 μg mL^{-1} and kanamycin (Sigma-Aldrich Sweden AB, Stockholm, Sweden) at 50 μg mL^{-1}.

2.2. Gene selection

All genes required for *trans*-resveratrol production were selected from the NCBI database. Here, we used *TAL* from *R. sphaeroides* (GenBank accession no. 77464988), and *4CL* and *STS* from *V. vinifera* (GenBank accession nos. AM428701.2 and DQ366301, respectively). To increase the expression levels in *Synechocystis*, we synthesized the nucleotide sequences of these genes using the optimized codon preference for *Synechocystis*, following the algorithm of GenScript Optimum GeneTM (GenScript USA Inc., Piscataway, NJ, USA). To increase the translation efficiency in cyanobacteria, the codon adaptation index of each gene was upgraded from approximately 0.60 in the native genes to above 0.85.

2.3. Plasmid constructions

To construct the expression vectors for the genes involved in *trans*-resveratrol biosynthesis, we designed forward