J Appl Phycol
DOI 10.1007/s10811-013-0113-5

Functional expression of an *Arabidopsis* p450 enzyme, *p*-coumarate-3-hydroxylase, in the cyanobacterium *Synechocystis* PCC 6803 for the biosynthesis of caffeic acid

Yong Xue · Yan Zhang · Stephen Grace · Qingfang He

Received: 2 May 2013 / Revised and accepted: 13 August 2013

Abstract Caffeic acid, which exhibits strong anticancer activities, is a natural phenolic compound found in small amounts in plants. Production of caffeic acid by bacterial systems is technically challenging due to difficulties in functionally expressing *p*-coumarate-3-hydroxylase (C3H), a cytochrome P450 enzyme that converts *p*-coumaric acid into caffeic acid. Here, we report for the first time that the cyanobacterium *Synechocystis* PCC 6803 is able to produce caffeic acid from *p*-coumaric acid upon heterologous expression of C3H. The *Arabidopsis thaliana ref8* gene, which encodes a C3H, was synthesized and codon-optimized for enhanced expression in *Synechocystis*. Expression of the synthetic *ref8* gene was driven by a native *psbA2* promoter and confirmed at the transcriptional and translational levels. This heterologous pathway enabled *Synechocystis* to produce caffeic acid at a concentration of ~7.2 mg L^{-1} from *p*-coumaric acid under oxygenic photosynthetic growth conditions. This study demonstrates that cyanobacteria are well suited for the bioproduction of plant secondary metabolites that are difficult to produce in other bacterial systems.

Keywords Caffeic acid · Cyanobacteria · P450 protein · Plant secondary metabolites · *Synechocystis* PCC 6803

Y. Xue and Y. Zhang contributed equally to this work.

Electronic supplementary material The online version of this article (doi:10.1007/s10811-013-0113-5) contains supplementary material, which is available to authorized users.

Y. Xue · S. Grace · Q. He
Department of Applied Science, University of Arkansas at Little Rock, 2801 South University Ave, Little Rock, AR 72204, USA

Y. Zhang · Q. He (✉)
High Tech Research Center, Shandong Academy of Agricultural Sciences, 202 Gongye North Road, Jinan, Shandong Province 250100, China
e-mail: qfhe@ualr.edu

Introduction

Caffeic acid (3,4-dihydroxycinnamic acid) is one of the phenylpropanoid secondary metabolites produced by plants in response to stress factors, such as pathogen infection, wounding, UV radiation, high light intensity, nutrient deficiency, and low temperature (Dixon 2001). Caffeic acid and its derivative, caffeic acid phenethyl ester, have numerous favorable biological and pharmacological properties, including strong antioxidant (Gulcin 2006), anticancer (Prasad et al. 2011), anti-inflammatory (Chao et al. 2009), and immunomodulatory activities (Park et al. 2004). With the increasing demand for phenylpropanoid dietary supplements, the current major production methods, i.e., chemical synthesis and plant extraction (Yoshimoto et al. 2005), exhibit significant drawbacks, such as low yield, high cost, and the use of toxic chemicals and catalysts. Therefore, microorganism production platforms are widely being explored as possible solutions for phenylpropanoid production, due to their rapid growth rates, potential low cost, and minimal impact on the environment.

The biosynthesis of caffeic acid in plants starts either with L-phenylalanine or L-tyrosine (Fig. 1). L-Phenylalanine is deaminated by phenylalanine ammonia-lyase (PAL) to form *trans*-cinnamic acid, and a hydroxyl group is then added to the 4-position of the aromatic ring by cinnamate 4-hydroxylase (C4H) to form *p*-coumaric acid. *p*-Coumaric acid can also be synthesized through direct deamination of tyrosine by tyrosine ammonia-lyase (TAL). The production of caffeic acid from *p*-coumaric acid requires *p*-coumarate-3-hydroxylase (C3H), which transfers another hydroxyl group to the 3-position of the

Published online: 07 September 2013

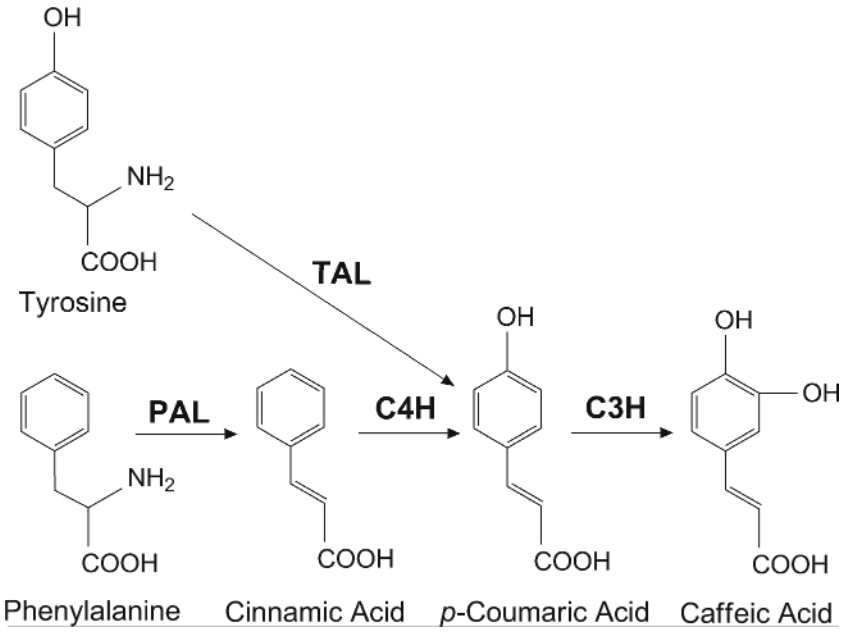

Fig. 1 The caffeic acid metabolic pathways. Abbreviations: *TAL* = tyrosine ammonia-lyase, *PAL* = phenylalanine ammonia-lyase, *C4H* = cinnamate-4-hydroxylase, and *C3H* = cinnamate-3-hydroxylase. The biosynthesis of caffeic acid starts either with tyrosine or phenylalanine. *p*-Coumaric acid, an intermediate of both pathways, accumulates and is further converted into caffeic acid in the presence of C3H

aromatic ring in *p*-coumaric acid (Rosler et al. 1997; Berner et al. 2006).

C3H is a cytochrome P450-dependent monooxygenase belonging to the CYP98 family (CYP98A3) (Franke et al. 2002). C3H has been identified as a NADPH- or flavin-dependent oxidase with mixed functions (Stafford and Dresler 1972; Kojima and Takeuchi 1989). The functional expression of C3H in bacterial systems has been problematic for the past 30 years due to its instability, low abundance, and membrane-bound nature (Kim et al. 2011). Native C3H was identified in *Saccharothrix espanaensis*; however, its application is limited due to low activity levels (Berner et al. 2006).

The photosynthetic cyanobacterium *Synechocystis* 6803 (GenBank accession no. BA000022.2) is an effective model system for dissecting photosynthetic and respiratory functions (Niyogi 1999). Its photosynthetic apparatus is similar to that of vascular plants. This provides us with an excellent system in which to manipulate growth conditions for optimal production of secondary metabolites and to study their antioxidant activities. Although cyanobacterial systems have not been extensively used for the production of industrial or pharmaceutical compounds or proteins, they may offer advantages in bioproduction over other systems due to their high level of protein expression and high yield of photosynthesis in low-cost growth media. Here, we explore the possibility of using cyanobacteria as a "cell factory" in which to produce caffeic acid through the expression of C3H under photosynthetic growth conditions.

Materials and methods

Codon usage optimization The nucleotide sequence of the *Arabidopsis ref8* gene was synthesized and optimized to enhance its expression based on the preferred codon usage of *Synechocystis*. Gene Designer software (DNA 2.0, Menlo Park, CA, USA) and a *Synechocystis* codon usage table from the Kazusa Codon Usage Database (Nakamura et al. 2000) were used to design the new gene. The codon-optimized gene (*sref8*) was synthesized by Integrated DNA Technologies, Inc. (Coralville, USA). The control *Arabidopsis ref8* gene was provided by Dr. Clint Chapple from Purdue University.

Plasmid construction To construct the plasmids containing an expression cassette for C3H (*ref 8* or *sref8*), a 1.25-kb kanamycin resistance cassette was excised from pUC4K (vector map: http://www.addgene.org/vector-database/4506/) using restriction enzymes *Bam*HI and *Pst*I, and inserted into pBluescript II SK+ (Stratagene, USA) between *Bam*HI–*Pst*I sites. Two flanking neutral site sequences in the *Synechocystis* genome (near *slr1285*) that target the gene cassette into an intergenic region of the genome were obtained by PCR using the following primers: 5′-ATCGGTACCGGCAATGC AATTAATTAAAAATGGC-3′ and 5′-ATCCTCGAGTCTA TTGTTGGAAGGTTGCTG-3′ for the upstream region (Chr:1880701-1881294), and 5′-ATCACTAGTGTGA AAAAATATTGACATTAAGAT-3′ and 5′-ATCCCGC GGGGAACCAGATTTTTAGGATGGG-3′ for the downstream region (Chr:1881310-1881916). The 600-bp upstream fragment and the 600-bp downstream fragment were inserted between *Kpn*I–*Xho*I sites and *Sac*II–*Spe*I sites of pBluescript II SK+, respectively. The 1.5-kb synthesized *sref8* gene (with a 3′ *FLAG* tag) was inserted into the *Eco*RI–*Pst*I sites of pBluescipt II SK+. A *psbA*2 promoter fragment was amplified from the *Synechocystis* genome using the primers 5′-TCAGTCGACGGTATATGGATCATAATTGTATGC-3′ and 5′-TCAGAATTCTTGGTTATAATTCCTTATGTATTTG-3′

and inserted into the *Eco*RI–*Sal*I sites of pBluescript II SK+. 5ST1T2, a transcriptional terminator, was amplified from pBTac1and inserted into the plasmid between the *Pst*I–*Sma*I sites.

Synechocystis culture conditions and transformation *Synechocystis* sp. PCC 6803 was grown in BG-11 (Rippka et al. 1979) liquid medium at 30 °C until an OD_{730} of 0.5–0.7 was reached. The cell culture was centrifuged at 1,378×*g* for 10 minutes, and cells were resuspended in fresh BG-11 liquid medium to an OD_{730} of 10. Two micrograms of plasmid DNA was mixed with 400 μL cells in a glass tube and incubated at 30 °C for 6 h. Cells were then spread on a sterile Nuclepore Track-Etch Membrane (Whatman, USA) on top of a BG-11 agar plate. After 24 h, the membrane was transferred to a new BG-11 plate containing 5 μg mL^{-1} kanamycin and incubated at 30 °C for about 2 weeks until colonies emerged. A single colony was streaked onto a new BG-11 plate with 25 μg mL^{-1} kanamycin to allow segregation to occur. Insertion and segregation of *sref8* in the genome was confirmed by PCR analysis.

Reverse transcription PCR Total RNA was isolated from 100-mL *Synechocystis* sp. PCC 6803 cultures at $OD_{730}\approx0.5$, as previously described (He and Vermaas 1998). Residual genomic DNA was removed by incubating the RNA solution with 27 units of RNase-free DNase I (Qiagen, USA) for 10 min at 25 °C. DNase-treated RNA were normalized to contain equivalent amounts of total RNA and used in a reaction containing 10 ng L^{-1} random hexamers and 0.5 mM deoxynucleotide triphosphates. After incubation at 65 °C for 5 min and 4 °C for 1 min, 1× first strand buffer, 5 mM of DTT, 200 units of SuperScript III Reverse Transcriptase (Invitrogen, USA), and 40 units of RNaseOUT Recombinant RNase inhibitor (Invitrogen) were added to the reaction. The reactions were incubated at 25 °C for 5 min and then at 50 °C for 50 min followed by 15 min of incubation at 70 °C to denature RNA secondary structure. The resultant cDNA was amplified by PCR using the following gene-specific primers: 5′-AAA CCCCCGCGTGCAACAAAAAGT-3′ and 5′-TGGAGC GGTGGGGCAACATCAAG-3′, for amplification of *sref8*, and 5′-AAGGTTTCCTCAGGCTGGTT-3′ and 5′-CGCGTCGATGTGAACTCT-3′, for amplification of the 16S rRNA gene, which was used as a loading control. The PCR condition was 1 cycle of predenaturation at 95 °C for 30 sec; 30 cycles of denaturation at 95 °C for 30 sec, annealing at 56 °C for 1 min, and elongation at 68 °C for 1 min; and 1 cycle of postelongation at 68 °C for 5 min. The amplified fragments were separated on 0.8 % agarose gels, and the bands were visualized by ethidium bromide staining.

Cell extract and thylakoid membrane preparation *Synechocystis* cultures were grown at 30 °C in BG-11 medium with the addition of 5 mM glucose and 25 μg mL^{-1} kanamycin until an OD_{730} of 0.6–0.8 was reached. Cells were washed with and resuspended in thylakoid buffer (20 mM MES/NaOH (pH 6.4), 5 mM $MgCl_2$, 5 mM $CaCl_2$, 20 % glycerol (vol/vol), 1 mM freshly made phenylmethylsulfonyl fluoride, and 5 mM benzamidine) to a final volume of 0.6 mL. The cell suspension was mixed with 0.6-mL glass beads (diameter 0.1 mm, BioSpec Products, USA) prewetted with thylakoid buffer, and shaken in a MiniBeadBeater (BioSpec Products, USA) three times for 30 s at 2-min intervals on ice. After centrifugation at 3,000×*g* for 10 min to remove unbroken cells and cell debris, the cell suspension was collected as total cell extract for SDS-PAGE analysis or was centrifuged for 15 min at 50,000×*g* to sediment the thylakoids. The pellet of thylakoid membranes was washed with 2 mL thylakoid buffer and homogenized in the same buffer to a volume of 150 μL.

Extraction of caffeic acid from culture media *Synechocystis* was cultured in BG-11 medium at 30 °C until an OD_{730} of ~0.7. Cells were concentrated by centrifugation at 1,378×*g* for 10 minutes and resuspended in BG-11 medium at 1/10 of the original volume. Then, 0.5 μM of *p*-coumaric acid was added to the culture, and samples were collected after 3 days. Four milliliters of culture was centrifuged at 18,700×*g* for 2 min to remove the cells. The supernatant (medium) was transferred to a new tube, and the pH was adjusted to 5.0 with 1 N HCl before it was stored at −20 °C overnight. The medium was extracted twice with an equal volume of ethyl acetate, and the combined extract was dried under a continuous flow of nitrogen gas. The residue was resuspended in 80 % methanol and used for HPLC and LC/MS analysis.

HPLC and LC/MS analysis of caffeic acid production HPLC was carried out by injecting 10 L of sample into a Thermo HPLC UV6000 system (Thermo Fisher Scientific, USA) attached to a photodiode array detector with a Grace Prevail C18 column (4.6 mm×250 mm, 5 μm) (Alltech Associates, USA). Caffeic acid was eluted with 0.5 % phosphoric acid (A) and 100 % methanol (B) at a flow rate of 1 mL min^{-1}. The following solvent ratios (A/B) were used: 95/5 from 0 to 2 min, 60/40 from 2 to 4 min, a gradient from 60/40 to 20/80 from 4 to 20 min, 10/90 from 20 to 22 min, and back to 95/5 from 22 to 26 min. *p*-Coumaric acid and caffeic acid peaks were identified by comparison with the retention time and UV/vis spectrum of standards (Sigma-Aldrich, USA) and were verified by mass spectrometry. LC/MS mass spectrometry was carried out using an Agilent 1100 HPLC-MSD-VI Ion Trap mass spectrophotometer with an electrospray ionization source. The same column and mobile phase conditions were used as above for HPLC analysis, except that the phosphoric acid was replaced with 0.1 % formic acid and the flow rate was reduced to 0.8 mL min^{-1}. The negative ion values of standards are as follows: *p*-coumaric acid (m/z 163.1) and caffeic acid (m/z 179.9).

SDS-PAGE and immunoblot analysis The protein concentrations of cell extracts or thylakoids were determined using the Bio-Rad assay (Bio-Rad Laboratories, USA). SDS-PAGE was performed using an 8 %–16 % gradient resolving gel with 6 M urea and a 5.5 % stacking gel containing 2 M urea. Cell extracts or thylakoids were incubated with sample loading buffer (60 mM Tris–HCl, pH 6.8, 2 % SDS, 5 % 2-mercaptoethanol, 25 % glycerol, and 0.1 % bromophenol blue) at 37 °C for 20 min before loading onto the gel. A total of 20 μg of protein was loaded per lane. After electrophoresis, the gel was either stained with Coomassie Brilliant Blue R250 or blotted onto a nitrocellulose membrane using the Bio-Rad Trans-Blot system (Bio-Rad Laboratories, USA), according to the standard procedure. The membrane was probed with specific primary antibodies (anti-C3H antibody from Dr. Michael Sullivan and anti-FLAG antibody from Sigma-Aldrich) and alkaline phosphatase-conjugated secondary antibodies (Sigma-Aldrich, USA). Cross reactions of proteins and antibodies were visualized using the BCIP/NBT Kit (Invitrogen, USA).

Results

Codon optimization and genetic engineering of Synechocystis The caffeic acid synthetic enzyme C3H used in this study is derived from *Arabidopsis thaliana*, in which the codon usage differs from that in *Synechocystis*. A new version of this gene sequence with codon usage optimized for *Synechocystis* was designed and synthesized (Fig. S1). Codons that are rarely used in *Synechocystis* were changed to those that are used more frequently, and the AT/GC ratio was adjusted to that of the host based on the codon usage table derived from the *Synechocystis* genomic sequence (Nakamura et al. 2000). Other factors affecting gene expression efficiency were also taken into consideration, such as mRNA secondary structure, repeat sequences, and restriction sites that may interfere with cloning. The differences in codon frequency of the native and modified *ref8* genes are plotted in Fig. S2. For the native gene, some of the codons show a low usage frequency (less than 8 %). These codons were modified in the codon-optimized gene, so that all codons present have a high usage frequency (above 23 %).

The codon-optimized *sref8* gene tagged with the FLAG octapeptide coding sequences at the 3′ end was sandwiched between the *Synechocystis psbA2* promoter and *Escherichia coli* 5S t1t2 terminator. This new version of the *sref8* gene, together with a kanamycin resistance cassette, was placed between the upstream and downstream regions of a neutral site (near *slr1285* in the genome). The resulting plasmid (Fig. 2a), harboring *sref8*, was used to transform wild-type *Synechocystis*, and *sref8* was integrated into the genome at the neutral site through double crossover homologous recombination. Because *Synechocystis* 6803 has eight to ten copies of its genome, we intend to segregate *sref8* throughout the chromosome. We streaked positive transformant colonies on a plate with high-concentration kanamycin (25 μg mL^{-1}). Only cells containing multiple copies of kanamycin resistance cassette can bear large amount of antibiotics and grow. The insertion of *sref8* in the *Synechocystis* chromosome and its complete segregation were further verified by PCR using genomic DNA from the *Synechocystis* transformant as template (Fig. 2b). After subculturing the transformant on selective medium, all wild-type copies of the neutral sites were replaced with the *sref8* gene locus (Fig. 2c).

Growth rates of the *sref8* transformant and wild-type strains were measured in BG-11 medium at 30 °C. The growth of the transformant strain was found to be similar to that of wild-type cells (doubling time ~10 h). This result indicates that the endogenous expression of C3H protein and the interruption of the neutral site do not have a negative effect on the growth or cell physiology of *Synechocystis*.

Expression analysis of sref8 in Synechocystis We analyzed the transcription of *sref8* in the transformed strain using reverse transcriptase PCR. Total RNA was prepared, and PCR using *sref8*-specific primers and first strand cDNA as template was performed. *sref8* transcript was detected as shown in Fig. 3 (lane M+RT). No amplification product was seen in the negative controls, which either used wild-type *Synechocystis* genomic DNA as template (Fig. 3, lanes W+RT, W−RT) or lacked reverse transcriptase, but contained *sref8* mRNA as template (Fig. 3, lane M−RT). These results confirmed that there is no genomic DNA in the mRNA preparations.

To study C3H protein expression in the *sref8* mutant strain, we performed SDS-PAGE and immunoblot analyses using thylakoid membrane fractions from the wild-type strain and *sref8* transformant. Immunoblot analysis was performed using anti-C3H polyclonal antibody and anti-FLAG monoclonal antibody. Specific immunoreactions between the antibodies and C3H (predicted size 58 kDa) in the *Synechocystis* transformant were visualized (Fig. 4a, b; lane M). Immunoblot analysis of the thylakoid fraction from the wild-type *Synechocystis* strain did not show any specific immunoreactivity (Fig. 4a, b; lane WT). In the SDS-PAGE analysis, we also observed a clear band at 58 kDa in the thylakoid membrane of the transformant, which was absent in that of the wild type. Therefore, C3H was expressed at a relatively high level in the *Synechocystis* transformants.

Production of caffeic acid by genetically engineered Synechocystis Next, we tested the activity of the heterologously expressed C3H by measuring the production of caffeic acid in the culture medium following the addition of *p*-coumaric acid,

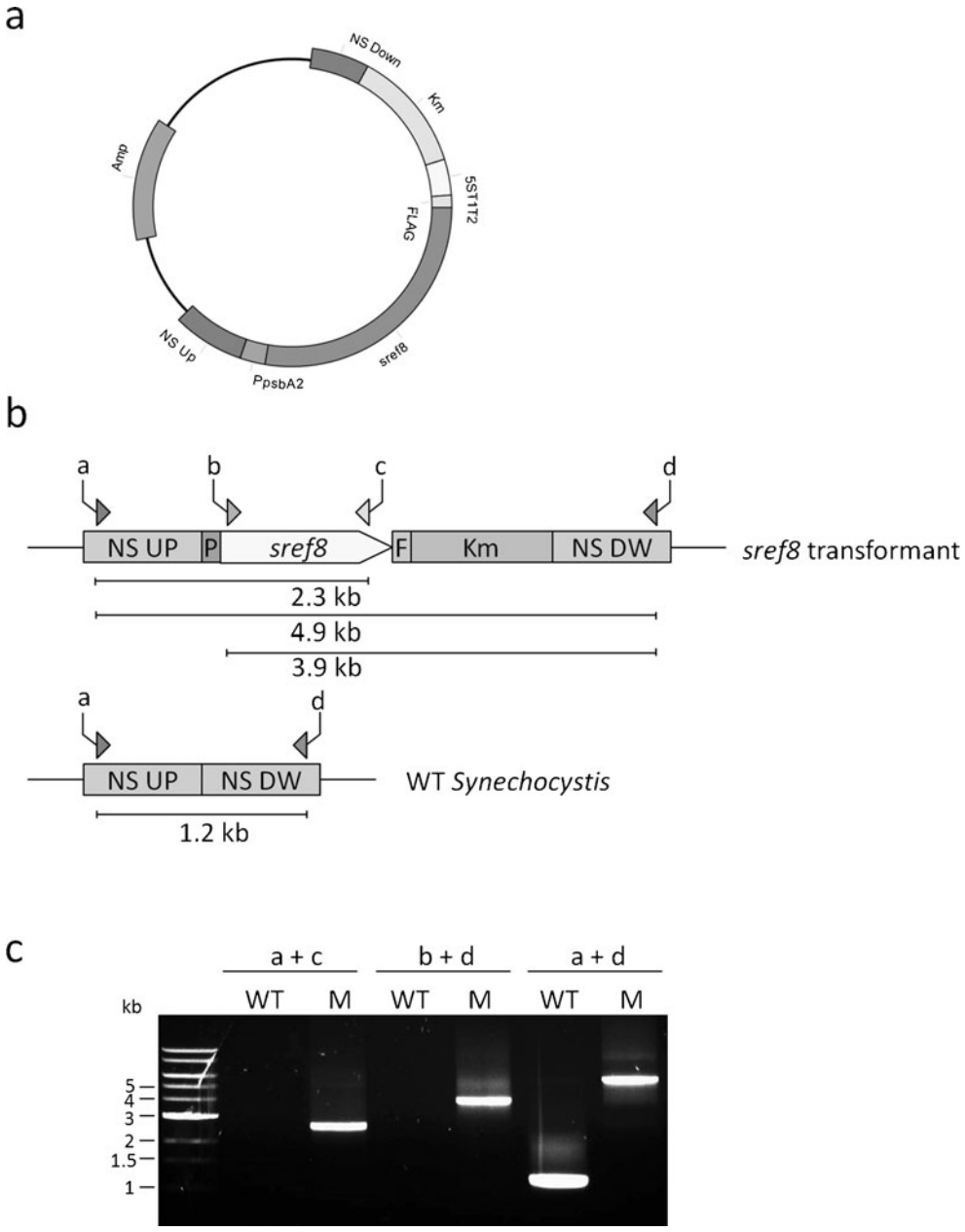

Fig. 2 Plasmid construction and transformation for *sref8* expression in *Synechocystis*. **a** *sref8* and a 3′ *FLAG* tag were cloned into the pBluescript SK(+) plasmid. The *psbA2* promoters, kanamycin resistance cassette (*Km*), upstream and downstream regions of the neutral site, and *E. coli* 5S t1t2 terminator were cloned into the plasmid as well. *Amp* ampicillin resistance cassette. **b** Simplified scheme of the wild-type and mutant *Synechocystis* with *sref8* inserted in the genome. Primers used for PCR screening are indicated, and the expected sizes of PCR products are shown as *lines* beneath each map. *NS UP* upstream region of the neutral site, *NS DW* downstream region of the neutral site. **c** PCR screening to test for insertion of *sref8* in the mutant. Lanes: *WT* wild type, *M sref8* mutant. The positions of the primers (*a*, *b*, *c*, and *d*) used for screening are indicated by *arrows* in **b**. Primers are *a*: 5-GGTACCGGCAATGCAATTA ATTAAAAATGGC-3′, *b*: 5′-GCAAGAATTCATGT CCTGGTTTTTGATTGC-3′, *c*: 5′-TCACTCGAGTTACATAT CGTAGGGCACGCGTTT-3′, and *d*: 5′-TCCGCGGAACCAGA TTTTTAGGATGGG-3′

the substrate of the C3H enzyme. Both the *sref8* transformant and the wild-type strain were cultured under normal light conditions, and the culture media were extracted for LC/MS analysis. The HPLC profile of the transformant has a caffeic acid peak at a retention time of 19.6 minutes (Fig. 5, a), which is absent in that of the wild type (Fig. 5, b and e). Further identification of this compound was performed using mass spectrometry. A compound with an *m*/*z* value of 179.9 (Fig. 5, f), which is identical to the caffeic acid standard, was detected only in the transformant.

Comparison of ref8 and sref8 transformants To study the effect of codon optimization on gene expression, thylakoid membranes from the wild type, *ref8* transformant, and *sref8* transformant at OD_{730} ~0.7 were isolated and analyzed by SDS-PAGE. The total protein concentration of each sample was measured, and 20 μg of protein was subjected to SDS-PAGE. Immunoblot analysis was performed using anti-C3H polyclonal antibodies. As shown in Fig. 4d, both the *ref8* and *sref8* transformants had detectable levels of C3H protein. However, the codon-optimized gene expressed the protein at much higher levels than the original gene.

Furthermore, we compared the yield of caffeic acid produced by transformant *Synechocystis* harboring *ref8* or *sref8* (Fig. 5, g). After incubating the *ref8* and *sref8* transformant cultures under normal light at 30 °C, we extracted the culture media for LC/MS

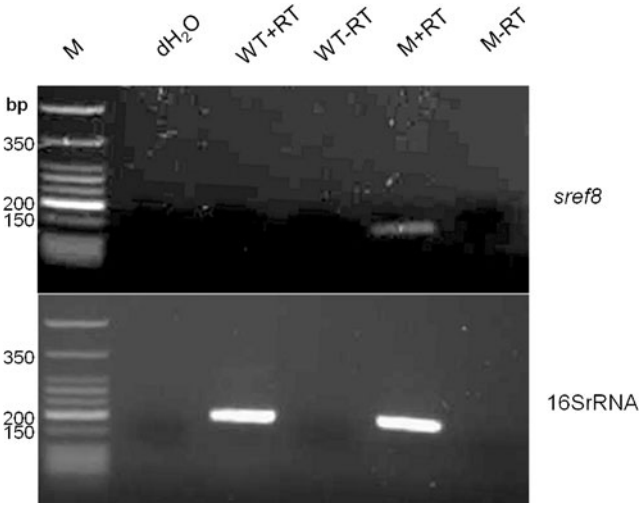

Fig. 3 RT-PCR analysis of the *sref8* transformant. Total RNA of the *sref8 Synechocystis* transformant was prepared for reverse transcriptase PCR. *Top panel*: PCR analysis using primers which bind to *sref8*. *Bottom panel*: PCR analysis using primers that bind to cDNA from 16S rRNA, which is used as a loading control. Lanes: *M* DNA ladder; dH_2O negative control for PCR using water as template; *WT+RT* RT-PCR using RNA from wild-type *Synechocystis* as template for the reverse transcription; *WT−RT* as for WT+RT, but with no reverse transcriptase in the reaction; *M+RT* RT-PCR using RNA from the *Synechocystis* mutant transformed with *sref8* as template for the reverse transcription; and *M−RT* as for M+RT, but with no reverse transcriptase in the reaction

analysis. The amounts of caffeic acid produced were determined by comparison with the peak area of a known quantity of caffeic acid standard. After codon optimization, the yield of caffeic acid increased from 5 to 7.2 mg L^{-1}.

Discussion

In this report, a genetically modified cyanobacterium was used as a host to express a plant cytochrome P450 protein, C3H, for the production of caffeic acid from *p*-coumaric acid. To our knowledge, this is the first report of the successful heterologous and functional expression of this type of protein in the cyanobacterium *Synechocystis* sp. PCC 6803. Cyanobacteria are emerging as a useful tool in the field of bioproduction. There have been several reports of cyanobacterial species being engineered to produce valuable compounds, including sugars (Niederholtmeyer et al. 2010), isoprene (Lindberg et al. 2010), alcohols (Deng and Coleman 1999), and alkanes (Reppas and Ridley 2011). By using photosynthetic cyanobacteria for bioproduction, the cost of carbohydrate feedstocks, which are required for the growth of nonphotosynthetic microorganisms, can be eliminated.

Caffeic acid is a natural phenolic compound derived from the plant phenylpropanoid pathway. Currently, the industrial production of caffeic acid either involves the chemical or enzymatic hydrolysis of caffeoylquinic acid derivatives or extraction from plant sources, such as coffee beans (Yoshimoto et al. 2005). These production approaches are either associated with environmental pollution or high cost due to the low level of caffeic acid expression in plants. This work shows that caffeic acid can be produced by *Synechocystis*, a photosynthetic microbe, through the heterologous expression of C3H and the addition of *p*-coumaric acid in the growth medium, in a system driven by cellular photosynthesis.

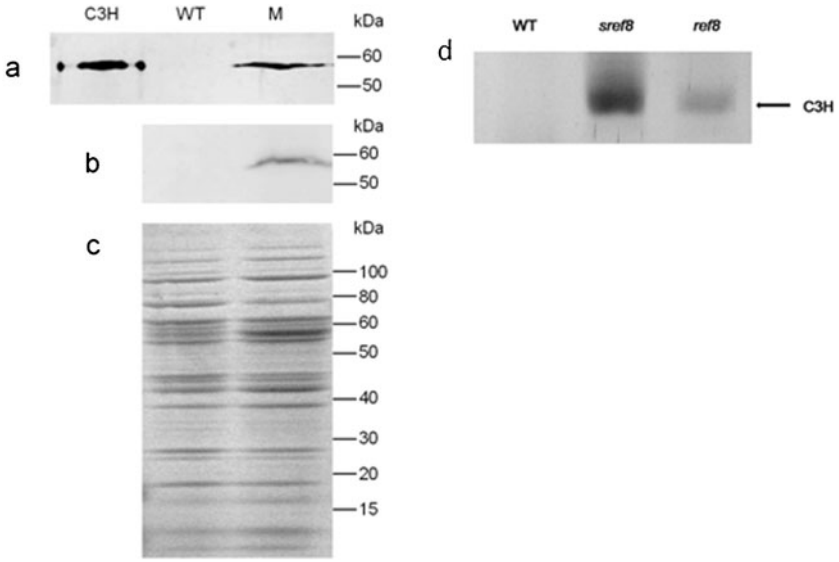

Fig. 4 Protein expression analysis of *sref8* in the *Synechocystis* transformant. **a** Immunoblot analysis using polyclonal anti-C3H antibody. **b** Immunoblot analysis using monoclonal anti-FLAG antibody. **c** Coomassie blue-stained SDS-PAGE profile of proteins. Lanes: *C3H* purified C3H protein (positive control), *WT* crude extracts from wild-type *Synechocystis*, *M* crude extracts from the *sref8*-expressing *Synechocystis* transformant. **d** Immunoblot analysis of wild-type *Synechocystis* and the *ref8* and *sref8* transformants. Total cell extracts of wild-type *Synechocystis* and *ref8* and *sref8* transformants were fractionated by SDS-PAGE, blotted to nitrocellulose membrane, and probed with anti-C3H antibody

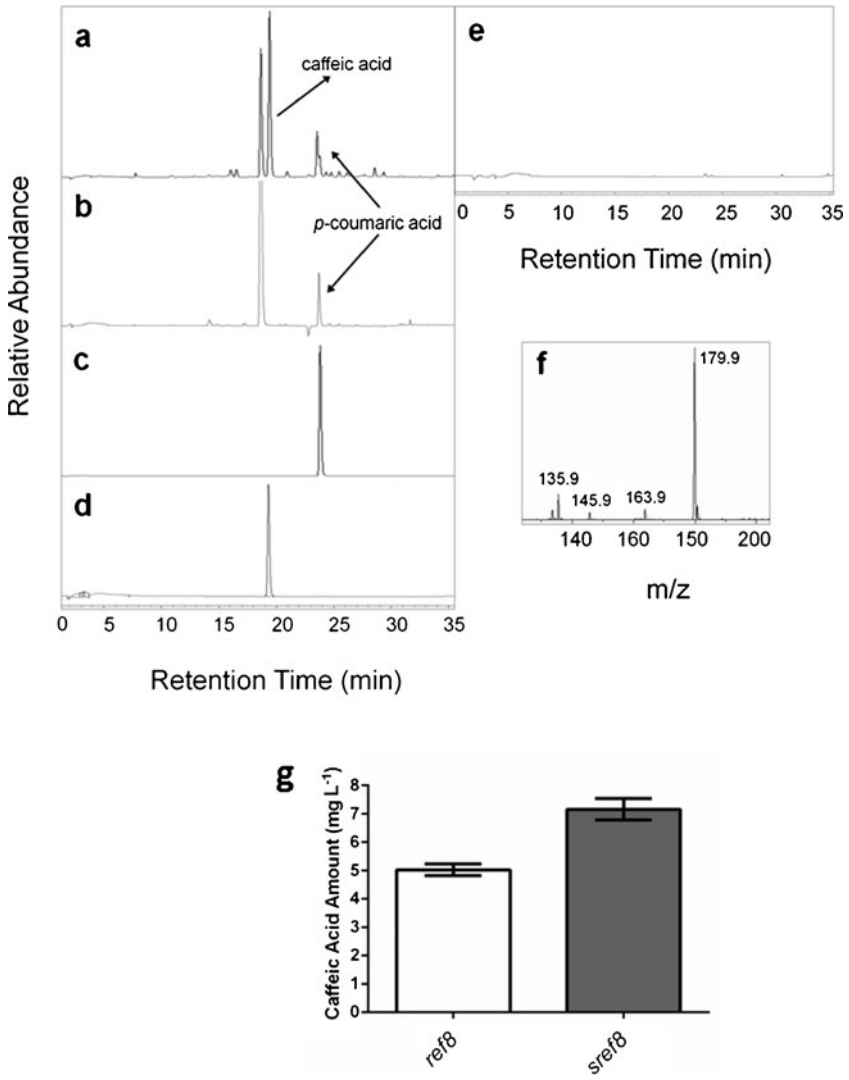

Fig. 5 LC/MS analysis of caffeic acid production. *Synechocystis* cells harboring *sref8* were incubated in BG-11 medium at 30 °C. The culture medium was extracted with ethyl acetate and subjected to LC/MS analysis. *a* HPLC profile of medium extract from the transformed *Synechocystis* strain grown in the presence of *p*-coumaric acid. *b* HPLC profile of medium extract from the wild-type *Synechocystis* 6803 strain with the addition of *p*-coumaric acid. *c* *p*-coumaric acid standard (MW 164). *d* caffeic acid standard (MW 180). *e* HPLC profile of medium extract from the wild-type *Synechocystis* 6803 strain without the addition of *p*-coumaric acid. *f* The mass spectrum of the peak at 19.6 min (caffeic acid) in *a*. *g* caffeic acid production yield of *Synechocystis* *ref8* and transformants. Culture media were extracted and quantified by HPLC analysis. *Error bars* represent one standard deviation for triplicate assays

There are several reasons why cyanobacteria are an appropriate choice of system for the production of caffeic acid and similar compounds.

A reaction in caffeic acid biosynthesis is catalyzed by a cytochrome P450 hydroxylase. Most of the proteins in this family can only be correctly assembled in eukaryotic systems. However, cytochrome P450-like proteins were found to exist ubiquitously in cyanobacteria. *Nostoc punctiforme* PCC 73102, a filamentous cyanobacterium, even has nine P450-like genes. One P450 gene has been reported in *Synechocystis* sp. PCC 6803: *cyp120A1* (*slr0574*). Although the characteristics of its redox partners remain unknown, it was shown to participate in retinoid metabolism (Ke et al. 2005). This suggests that cyanobacteria are able to assemble cytochrome P450 proteins.

Furthermore, as a photosynthetic organism, *Synechocystis* has an abundance of NAD(P)H (cofactor for P450s), and the level can be further increased by photomixotrophic growth. This provides the basis for electron transfer to P450s and the functional expression of C3H protein in *Synechocystis*.

It is challenging to express plant P450s in *E. coli*, because this microorganism lacks a developed membrane system, and most eukaryotic P450s are membrane bound. Several plant P450 proteins have been expressed in *E. coli* (Chen et al. 2003; Hansen et al. 2001); however, none of them are soluble or functional. We attempted to express *ref8* in *E. coli*, but did not detect any functional C3H protein (data not shown). In contrast to other prokaryotes, which lack internal membrane systems, cyanobacteria (e.g., *Synechocystis*) contain a multifunctional, intracellular membrane system called thylakoids, on which electron transport for both respiration and photosynthesis takes place (Melis 1999).

The extra peak at 18.8 min in both wild-type and *sref8*-expressing *Synechocystis* (Fig. 5, a and b) upon the addition of *p*-coumaric acid has an *m*/*z* of 121, which is predicted to be 4-vinylphenol. This could be generated from the decarboxylation

of *p*-coumaric acid by cinnamate decarboxylase. While 4-vinylphenol is a valuable compound found in wine and beer, this side reaction reduces the availability of *p*-coumaric acid as substrate for the biosynthesis of caffeic acid. To improve the titer of caffeic acid production, strategies could be designed to minimize the decarboxylation step. These include knocking out genes that encode potential decarboxylases from the *Synechocystis* genome or adding a decarboxylase inhibitor (such as catechin) to the culture (Chatonnet et al. 1993).

We successfully expressed C3H in *Synechocystis* sp. PCC 6803 and produced caffeic acid in this heterologous system at a yield of 7.2 mg L^{-1}. The caffeic acid is excreted into the media, which simplifies extraction, concentration, and purification. Cyanobacteria have additional advantages that make them an attractive system for this purpose, including ease of genetic manipulation, relatively fast growth rates, and a similar photosynthetic apparatus to vascular plants. These features of cyanobacteria make them highly suitable for the production of plant secondary metabolites, a process that is not possible in other microbial systems.

Acknowledgments This work was supported by the National Science Foundation (grant no. MCB1120153), Shandong Province "Taishan Scholar" Foundation (no. tshw20091014), and by the Arkansas P3 Center (pilot seed grant P3-203). This paper is dedicated to the memory of Ms. Jing Zhang, a former master's student in the laboratory, who contributed significantly to the project. The authors thank Dr. Michael Sullivan for the anti-C3H antibody (which was obtained through a Material Transfer Agreement).

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