J|A|C|S
COMMUNICATIONS

Published on Web 09/20/2006

Using Unnatural Protein Fusions to Engineer Resveratrol Biosynthesis in Yeast and Mammalian Cells

Yansheng Zhang,[†] Song-Zhe Li,[‡] Jia Li,[†] Xiangqing Pan,[†] Rebecca E. Cahoon,[†] Jan G. Jaworski,[†] Xuemin Wang,[†,§] Joseph M. Jez,[†] Feng Chen,[‡] and Oliver Yu[†,*]

Donald Danforth Plant Science Center, St. Louis, Missouri 63132, Departments of Medicine and Cell Biology & Physiology, Washington University School of Medicine, St. Louis, Missouri 63110, and Department of Biology, University of Missouri, St. Louis, Missouri 63121

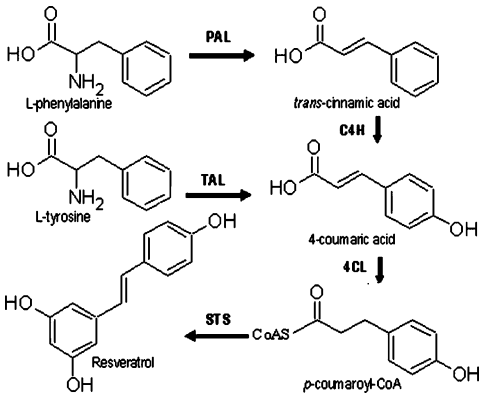
Received March 31, 2006; E-mail: oyu@danforthcenter.org

The formation of macromolecular complexes in biosynthetic pathways improves metabolic efficiency either by colocalizing enzymes or by the channeling of metabolites.[1] For metabolic engineering purposes, linking genes together to generate a functional fusion protein offers an attractive strategy for increasing product yields. The use of fusion proteins also reduces the number of vectors in a heterologous expression system, which simplifies the reconstitution of metabolic pathways. If naturally occurring protein complexes enhance biosynthetic efficiency, then generating unnatural protein fusions between sequential biosynthetic enzymes may yield similar results. To test this hypothesis, we focused on engineering the biosynthesis of the plant polyketide resveratrol (*trans*-3,5,4′-trihydroxystilbene).

Resveratrol is produced by certain plants, including grapes and peanuts, in response to UV-irradiation or fungal infection.[2] It is also a major health-promoting compound in red wine and functions as an antioxidant.[3] Consumption of resveratrol significantly extends the life span of yeast, fruit fly, round worm,[4] and fish;[5] however, the mechanism of action is unclear.[6] Resveratrol biosynthesis (Scheme 1) begins with the deamination of phenylalanine by phenylalanine ammonia lyase (PAL) to produce cinnamic acid, which is then hydroxylated by cinnamate-4-hydroxylase (C4H) to form 4-coumaric acid. This product is attached to CoA by 4-coumarate-CoA ligase (4CL). Next, stilbene synthase (STS) condenses 4-coumaroyl-CoA with three molecules of malonyl-CoA to form resveratrol. In heterologous expression systems, tyrosine ammonia lyase (TAL) can replace PAL and C4H by producing 4-coumaric acid from tyrosine.[7] Previously, we and others demonstrated that *Sacchromyces cerevisiae* and *Escherichia coli* can produce flavonoids by introducing multiple enzymes of the phenylpropanoid pathway and feeding the transgenic organisms appropriate substrates.[8] For example, *S. cerevisiae* coexpressing 4CL and STS produces resveratrol when fed 4-coumaric acid;[9] however, only low levels of product (<1.5 ng mL^{-1}) were obtained. To explore possible medical and biotechnology applications, such as engineering tissue-specific in situ biosynthesis of therapeutic compounds, we engineered the de novo resveratrol biosynthesis in mammalian cells by coexpressing TAL and a 4CL::STS fusion protein.

To reconstitute the resveratrol biosynthetic pathway, TAL from *Rhodobacter sphaeroides*, 4CL from *Arabidopsis thaliana*, and STS from *Vitis vinifera* were cloned by PCR. Each gene was subcloned into a yeast expression vector with a different selection marker and placed under control of a galactose-inducible promoter. In vivo

Scheme 1



activity of each enzyme was confirmed using established methods (see Supporting Information).

First, *S. cerevisiae* strain WAT11 was cotransformed with the 4CL and STS constructs. After galactose induction, gene expression was confirmed by RT-PCR analysis (not shown). After the feeding of 4-coumaric acid (10 h; 12 μg mL^{-1}), the culture media and yeast cells were separated by centrifugation, and the organic compounds extracted from each using ethyl acetate. Dried extracts were resuspended in 80% methanol and analyzed by HPLC. In the cell extract, a new peak with the same retention time and UV spectrum as *trans*-resveratrol was observed (Figure S1). LC−MS analysis confirmed the product identity as resveratrol (Figure S2). Yeast transformed with either 4CL or STS alone did not produce resveratrol. Cotransformed yeast produced 0.65 μg mL^{-1} resveratrol, which is 446-fold higher than previous reported.[9] In addition, the extracts from yeast cell pellets showed resveratrol accumulation (6.0 μg mL^{-1}, fresh cell volume). In contrast to earlier work,[9] resveratrol was present as the aglycone. This difference likely results from the use of different yeast strains. Surprisingly, yeast also produced *cis*-resveratrol (9.8% of the product yield) (Figure S1B), as confirmed by LC-MS using the authentic standard (not shown). Although this was not reported previously,[9] this result is consistent with the in vitro activity of STS in grapes.[10]

Since protein−protein interactions may increase metabolic efficiency either by channeling intermediates between enzymes or by localizing two active sites in close proximity, an expression construct encoding a translational fusion protein of 4CL and STS (4CL::STS) was generated. The fusion protein consisted of the 4CL with its stop codon replaced by a three-amino acid linker, followed by STS. The 4CL::STS vector was transformed into the WAT11 yeast strain. After galactose induction, the cultures were fed 4-coumaric acid (10 h; 12 μg mL^{-1}) and analyzed as above. Expression of the 4CL::STS fusion protein increased resveratrol

[†] Donald Danforth Plant Science Center.
[‡] Washington University School of Medicine.
[§] University of Missouri.

10.1021/ja0622094 CCC: $33.50

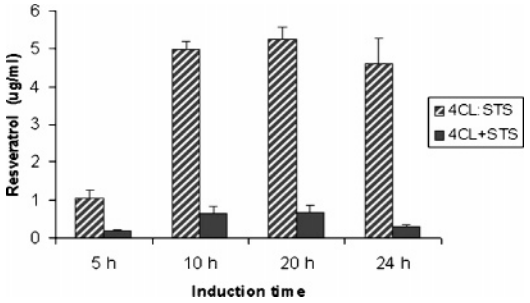


Figure 1. Resveratrol production is lower in the yeast strain cotransformed with 4CL and STS than in the yeast strain transformed with the 4CL::STS fusion protein. Error bar is the standard deviation for $n = 3$.

production in yeast by up to 15-fold compared to coexpression of 4CL and STS (Figure 1). Expression of the STS gene was similar in each yeast strain based on semiquantitative RT-PCR (Figure S3). After 20 h, yeast expressing the 4CL::STS fusion protein produced 5.25 μg mL^{-1} of resveratrol in the culture, which is 3,500-fold higher than previously reported.[9]

Because of the difficulty of expressing the TAL gene in yeast (not shown), we engineered the entire de novo resveratrol biosynthetic pathway into mammalian cells. We cloned the TAL (Figure S4) into a mammalian transfection vector under the control of a constitutive CMV promoter. The resulting vector produces a polycistronic expression unit with TAL and the hygromycin resistant gene (*hpt*) linked by an attenuated internal ribosome entry site (IRES). IRES allows simultaneous translation of TAL and *hpt* and ensures that the *hpt* gene is transcribed at a lower level than TAL, leading to high levels of TAL transcription when the transfected cells are treated with hygromysin.[11] Next, we cloned the 4CL::STS fusion gene into another transfection vector that contains a CMV promoter and a neomycin resistance cassette.[12]

The two constructs were cotransfected into human HEK293 kidney cells. The transfected cells were selected with both G418 and hygromycin for 4 weeks. Both transgenes were highly expressed in the cotransfected cells based on semiquantitative RT-PCR (Figure S5). Tyrosine or 4-coumaric acid was added to different batches of the transfected cells independently, and cultured for 2 days. The collected cells were resuspended in a potassium phosphate buffer (pH 7.0), sonicated, and extracted with ethyl acetate. The extracts were analyzed by HPLC and LC−MS. When fed with 4-coumaric acid (12 μg mL^{-1}), the cotransfected lines showed a resveratrol peak not found in the control at the correct retention time (Figure 2A, D) with an identical UV spectrum to the authentic standard. All the resveratrol produced accumulated inside the cells and was in the trans aglycone form, as confirmed by LC−MS analysis (Figure S6). In mammalian cells, 0.34 μg mL^{-1} (cell volume) resveratrol was produced. When fed with tyrosine, both 4-coumaric acid and *trans*-resveratrol were detected in the cotransfected lines, indicating that both TAL and the fusion protein are functional (Figure 2B,E). The production of resveratrol was estimated at 85 ng mL^{-1} (cell volume). Once again, all the resveratrol accumulated within the cells. Interestingly, without substrate feeding, the cotransfected cells synthesized 4-coumaric acid and *trans*-resveratrol, using phenylalanine and/or tyrosine that are present endogenously or in the cultured medium (Figure 2C,F). Without substrate feeding, the cells yielded 28.3 ng mL^{-1} of resveratrol. Comparison of the yields from these three experiments indicates that the supply of phenylalanine or tyrosine is limiting in mammalian cells. Taken together, introducing the TAL, 4CL, and STS genes into a mammalian cell line leads to de novo resveratrol biosynthesis.

Our results demonstrate that using unnatural fusion proteins for metabolic engineering offers a strategy for improving pathway

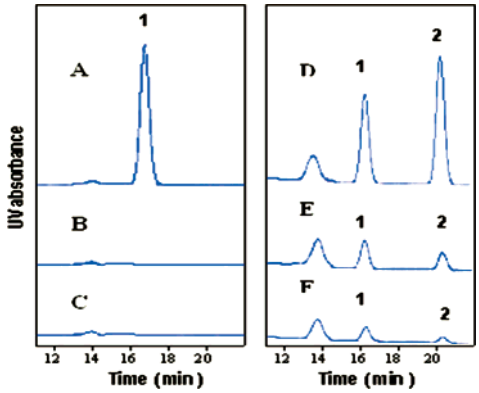


Figure 2. HPLC analysis of control HEK293 cells (A, B, and C) and TAL/4CL::STS cotransfected HEK293 cells (D, E, and F). A and D were fed with 4-coumaric acid; B and E were fed with tyrosine; C and F were not fed with any substrates. Peaks 1 and 2 are 4-coumaric acid and *trans*-resveratrol, respectively. All traces were from 290 nm UV profile.

yields by colocalizing 4CL and STS. Moreover, the engineering of a plant metabolic pathway in mammalian cells may provide additional opportunities and applications for medical and pharmaceutical research. For example, engineered resveratrol by de novo biosynthesis can conceivably improve cell replacement therapies by prolonging the life span of donor cells. β-Cell replacement therapy for type I diabetes using inlet transplantation showed tremendous promise in recent years, but is somewhat limited by the short life span of the transplanted β-cells.[13] Although there are considerable obstacles to overcome, donor β-cells engineered to produce resveratrol may survive longer, improving the efficacy and efficiency of this promising cell replacement therapy.

Acknowledgment. This work was supported by NSF (Grant MCB0519634), USDA (Grant 2005-05190), and MSMC (Grant 02-242) grants to O.Y. and NSF-DBI Grant 0521250 to X.W., J.G.J., and O.Y. We thank Peter Murphy for technical support.

Supporting Information Available: Experimental details and additional results. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Winkel, B. S. J. *Annu. Rev. Plant Physiol. Mol. Biol.* **2004**, *55*, 85−107.
(2) Schröder, J.; Schröder, G. *Z. Naturforsch.* **1990**, *45*, 1−8.
(3) Jang, M.; Cai, L.; Udeani, G. O.; Slowing, K. V.; Thomas, C. F.; Beecher, C. W.; Fong, H. H.; Farnsworth, N. R.; Kinghorn, A. D.; Mehta, R. G.; Moon, R. C.; Pezzuto, J. M. *Science* **1997**, *275*, 218−220.
(4) Howitz, K. T.; Bitterman, K. J.; Cohen, H. Y.; Lamming, D. W.; Lavu, S.; Wood, J. G.; Zipkin, R. E.; Chung, P.; Kisielewski, A.; Zhang, L. L.; Scherer, B.; Sinclair, D. A. *Nature (London)* **2003**, *425*, 191−196.
(5) Valenzano, D. R.; Terzibasi, E.; Genade, T.; Cattaneo, A.; Domenici, L.; Cellerino, A. *Curr. Biol.* **2006**, *16*, 296−300.
(6) Kaeberlein, M.; McDonagh, T.; Heltweg, B.; Hixon, J.; Westman, E. A.; Caldwell, S. D.; Napper, A.; Curtis, R.; DiStefano, P. S.; Fields, S.; Bedalov, A.; Kennedy, B. K. *J. Biol. Chem.* **2005**, *280*, 17038−17045.
(7) Kyndt, J. A.; Meyer, T. E.; Cusanovich, M. A.; Van Beeumen, J. J. *FEBS Lett.* **2002**, *512*, 240−244.
(8) (a) Ralston, L.; Subramanian, S.; Matsuno, M.; Yu, O. *Plant Physiol.* **2005**, *137*, 1375−1388. (b) Watts, K. T.; Lee, P. C.; Schmidt-Dannert, C. *ChemBioChem* **2004**, *5*, 500−507. (c) Jiang, H.; Wood, K. V.; Morgan, J. A. *Appl. Environ. Microbiol.* **2005**, *71*, 2962−2969.
(9) Becker, J. V.; Armstrong, G. O.; van der Merwe, M. J.; Lambrechts, M. G.; Vivier, M. A.; Pretorius, I. S. *FEMS Yeast Res.* **2003**, *4*, 79−85.
(10) Romero-Perez, A. I.; Ibern-Gomez, M.; Lamuela-Raventos, R. M.; de La Torre-Boronat, M. C. *J. Agric. Food Chem.* **1999**, *47*, 1533−1536.
(11) (a) Jang, S. K.; Davies, M. V.; Kaufman, R. J.; Wimmer, E. *J. Virol.* **1989**, *63*, 1651−1660. (b) Jang, S. K.; Krausslich, H. G.; Nicklin, M. J.; Duke, G. M.; Palmenberg, A. C.; Wimmer, E. *J. Virol.* **1988**, *62*, 2636−2643.
(12) (a) Boshart, M.; Weber, F.; Jahn, G.; Dorsch-Hasler, K.; Fleckenstein, B.; Schaffner, W. *Cell* **1985**, *41*, 521−530. (b) Nelson, J. A.; Reynolds-Kohler, C.; Smith, B. A. *Mol. Cell. Biol.* **1987**, *7*, 4125−9.
(13) Stock, P. G.; Bluestone, J. A. *Annu. Rev. Med.* **2004**, *55*, 133−56.

JA0622094