J. Microbiol. Biotechnol. (2014), 24(5), 614–618
http://dx.doi.org/10.4014/jmb.1401.01031

jmb

Construction of Artificial Biosynthetic Pathways for Resveratrol Glucoside Derivatives

Oksik Choi[1], Jae Kyoung Lee[1], Sun-Young Kang[1], Ramesh Prasad Pandey[2], Jae-Kyung Sohng[2], Jong Seog Ahn[1], and Young-Soo Hong[1]*

[1]*Chemical Biology Research Center, Korea Research Institute of Bioscience and Biotechnology, Ochang-eup 363-883, Republic of Korea*
[2]*Institute of Biomolecule Reconstruction, Department of Pharmaceutical Engineering, Sun Moon University, Asan-si 336-708, Republic of Korea*

Received: January 16, 2014
Revised: February 18, 2014
Accepted: February 20, 2014

First published online
February 25, 2014

**Corresponding author*
Phone: +82-43-240-6144;
Fax: +82-43-240-6158;
E-mail: hongsoo@kribb.re.kr

Supplementary data for this paper are available on-line only at http://jmb.or.kr.

pISSN 1017-7825, eISSN 1738-8872

Resveratrol, which is a polyphenolic antioxidant, is dose-dependent when used to provide health benefits, to enhance stress resistance, and to extend lifespans. However, even though resveratrol has therapeutic benefits, its clinical therapeutic effect is limited owing to its low oral bioavailability. An *Escherichia coli* system was developed that contains an artificial biosynthetic pathway that produces resveratrol glucoside derivatives, such as resveratrol-3-*O*-glucoside (piceid) and resveratrol-4′-*O*-glucoside (resveratroloside), from simple carbon sources. This artificial biosynthetic pathway contains a glycosyltransferase addition (YjiC from *Bacillus*) with resveratrol biosynthetic genes. The produced glucoside compounds were verified through the presence of a product peak(s) and also through LC/MS analyses. The strategy used in this research demonstrates the first harnessing of *E. coli* for *de novo* synthesis of resveratrol glucoside derivatives from a simple sugar medium.

Keywords: Artificial biosynthesis, resveratrol, glycosylation, piceid

Resveratrol can prevent, or delay, the progression of illnesses such as cancer, cardiovascular disease and ischemic injuries; it can also enhance stress resistance and extend the lifespans of various organisms from yeast to vertebrates [2, 7]. These effects have been observed despite its extremely low bioavailability and rapid clearance from the circulation [18]. However, administering higher doses of resveratrol in order to improve its efficacy might not be possible because toxic effects have been observed. Although resveratrol has therapeutic benefits, its water-insolubility limits its pharmacological exploitation in the healthcare industry [18]. A number of studies have suggested that piceid (resveratrol-3-*O*-glucoside), like resveratrol, may have similar bioactivities, such as anticarcinogenic effects, inhibition of platelet aggregation, antiinflammatory activity, and antioxidation activity [18]. Recently, it is also worth noting that piceid has higher scavenging activity against hydroxyl radicals than resveratrol *in vitro* [16]. Therefore, blocking the metabolism of resveratrol, developing resveratrol glucoside derivatives with improved bioavailability and solubility, or finding more potent compounds that mimic its effects are becoming increasingly important. Based on these reasons, some researchers are investigating the use of microbial cultures as *in vitro* biocatalysts to prepare resveratrol glucoside derivatives [15, 17], particularly in the production of piceid as a microbial metabolite of resveratrol from whole-cell suspensions of *Bacillus cereus* [5].

Resveratrol biosynthesis begins with the deamination of phenylalanine through phenylalanine ammonia lyase (PAL) in order to produce cinnamic acid, which is then hydroxylated by cinnamate-4-hydroxylase (C4H) to form *p*-coumaric acid. This product is attached to coenzyme A (CoA) via 4-coumarate-CoA ligase. Next, stilbene synthase (STS) condenses 4-coumaroyl-CoA, using three molecules of malonyl-CoA to form the resveratrol. Several types of glycosyltransferase have been demonstrated to be involved in the phenylpropanoid metabolism, but there have been

few reports on a specific glycosyltransferase to resveratrol [10, 12]. Although previous studies have already made resveratrol glucoside derivatives in microbe, the approaches were limited to the *in vitro* enzymatic reaction and the bioconversion of resveratrol by recombinant *Escherichia coli* [12, 15, 17]. Recently, the YjiC-homologous glycosyltransferases from other *Bacillus* species have been found to have flexible glycosyltransferase activities toward various benzoic compounds [1, 11, 14, 21]. It was already demonstrated that YjiC glycosyltransferase facilitates the *in vitro* glycosylation of the aromatic moiety of phenylpropanoid, such as apigenin, phlorectin, and several flavonols [1, 6, 11, 13, 14].

Meanwhile, it has been demonstrated that *E. coli* can produce resveratrol through the introduction of multiple enzymes in the phenylpropanoid pathway [4]. In this artificial expression system, tyrosine ammonia lyase (TAL) can replace PAL and C4H by producing *p*-coumaric acid from tyrosine. In this paper, the construction of an artificial biosynthetic pathway containing a UDP-glycosyltransferase gene (*yjiC*) addition with resveratrol biosynthetic genes is presented (Fig. 1). This *E. coli* system that contains an artificial biosynthetic pathway produces resveratrol glucoside derivatives, such as piceid (resveratrol-3-*O*-glucoside) and resveratroloside (resveratrol-4′-*O*-glucoside), from simple carbon sources. Although these compounds have been previously identified to be produced in various plants, we describe a different approach for their *de novo* synthesis in *E. coli* by engineering an artificial biosynthetic pathway.

In order to construct a plasmid that contains the artificial resveratrol glucoside biosynthetic pathway, our previously reported pET-TLkS cloning method was used [4]. The artificial resveratrol biosynthetic plasmids each contained a gene with their own T7 promoter, ribosome-binding site (RBS), and terminator sequence, as in the parental vectors. Although production of the resveratrol in *E. coli* using precursor (*p*-coumaric acid) conversion has been previously reported by other researchers [19, 20], the construction of the artificial biosynthetic pathway (pET-TLkS) was first reported as the *de novo* synthesis of resveratrol in *E. coli* using a simple sugar medium [4]. Here, the resveratrol-producing construct pET-opTLS was used, as in the parental vectors, that contain the codon-optimized *tal* gene of *Saccharothrix espanaensis* [9], cloned *ccl* gene of *Streptomyces coelicolor* [8], and codon-optimized *sts* gene of *Arachis hypogaea* [20]. For the production of resveratrol glucoside derivatives in *E. coli*, the only addition was the insertion of

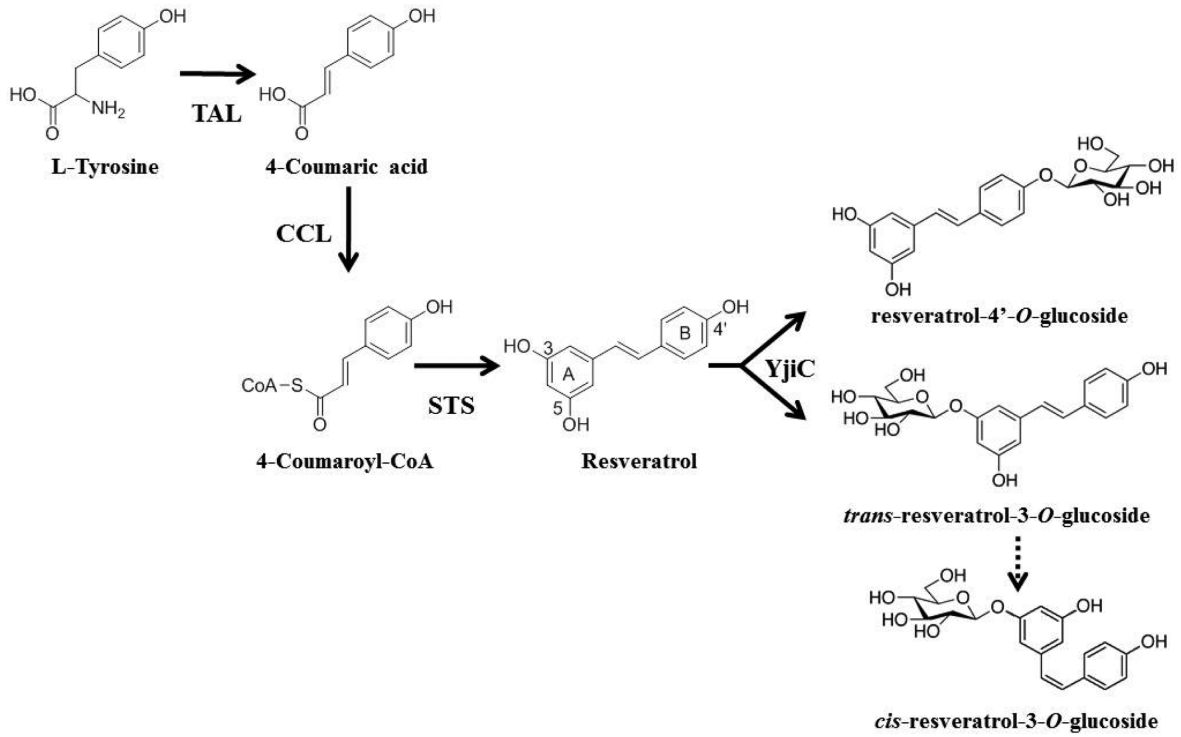

Fig. 1. Engineered biosynthetic pathways for the resveratrol glucoside derivatives starting from tyrosine in *E. coli*.
The artificial resveratrol biosynthetic pathway (pET-opTLS) only produces *trans*-resveratrol, as demonstrated in the HPLC. However, the *cis*-resveratrol-3-*O*-glucoside (*cis*-piceid) is produced after glycosylation in a long-time culture.

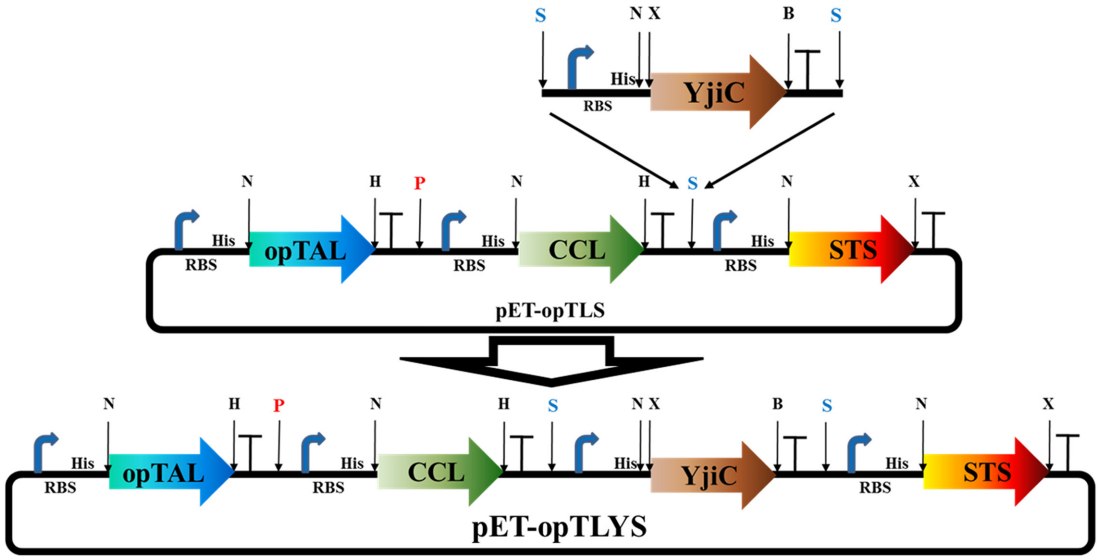

Fig. 2. Schematic representation of the artificial gene clusters used to produce the resveratrol glucoside derivatives.
All constructs contained the T7 promoter and RBS in front of each gene and the T7 terminator located in the rear of each gene. opTAL is a codon-optimized TAL enzyme. B, *Bam*HI; Bg, *Bgl*II; H, *Hind*III; P, *Pst*I; Nd, *Nde*I; P, *Pac*I; S, *Spe*I.

the glycosyltransferase gene (*yjiC*) in the pET-opTLS plasmid containing the resveratrol biosynthetic pathway (Fig. 2). The DNA fragment containing the promoter, YjiC coding region, and terminator was amplified using the pET302-YjiC plasmid, which is similar to the previously reported YjiC expression vector [13, 21]. The 1.2-kb DNA fragment containing the YjiC coding region was PCR-amplified using primers NSpe (5′-ACTAGTAGGTTGAGG CCGTTGAGCACCGCC-3′) and CSpe (5′-ACTAGTTCC TCCTTTCAGCAAAAAACCCCTC-3′) with pET302-YjiC as a template. The amplified fragments were digested with each restriction enzyme and cloned between the *Spe*I-digested pET-opTLS, which resulted in pET-opTLYS (Fig. 2).

Recombinant *E. coli* C41(DE3) strain harboring pET-opTLYS was cultured in modified M9 minimal medium supplemented with 15 g/l glucose as the sole carbon source and 25 g/l $CaCO_3$ [9]. Upon 1 mM IPTG induction, the secreted resveratrol glucoside derivatives in the culture medium were analyzed using high-performance liquid chromatography (HPLC) after 36 h. Twenty milliliters of culture was extracted with an equal volume of ethyl acetate. The ethyl acetate was dried in a vacuum, and the dried residue was resuspended in 400 µl of methanol. Twenty microliters of extract was applied to a SunFire C18 column (4.6 × 250 mm, 5 µm; Waters, UK) using a HPLC system (acetonitrile (CH_3CN)–H_2O (0.05% trifluoroacetic acid) 10% to 40% CH_3CN for 40 min, at flow rate of 1 ml/min; Dionex, USA) equipped with a photodiode array detector.

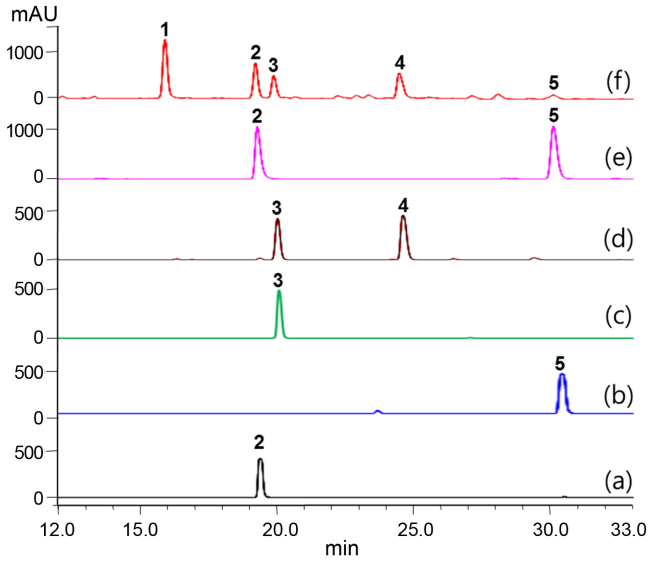

Fig. 3. Comparison of the HPLC profiles of the produced metabolites of *E. coli* that harbor pET-opTLS and pET-opTLYS.
The absorbance was monitored at 300 nm. (a) Authentic 4-coumaric acid; (b) authentic *trans*-resveratrol; (c) authentic *trans*-resveratrol-3-*O*-glucoside (piceid); (d) UV-treated authentic piceid (mixture of *trans*- and *cis*-piceid); (e) *E. coli* harboring pET-opTLS; and (f) *E. coli* harboring pET-opTLYS. Peak 1, resveratrol 4′-*O*-glucoside; peak 2, 4-coumaric acid; peak 3, *trans*-resveratrol 3-*O*-glucoside; peak 4, *cis*-resveratrol 3-*O*-glucoside; peak 5, *trans*-resveratrol.

Comparison of the fermentation products of the *E. coli* cells harboring pET-opTLYS and pET-opTLS revealed that three

new peaks were reproducibly detected in the engineered strain (Fig. 3). These compounds were further analyzed using liquid chromatography-mass spectrometry (LC-MS) in positive-ion mode. The LC-MS was performed using a LTQ-XL linear ion trap mass spectrophotometer (Thermo Electron, USA) equipped with an electrospray ionization (ESI) source. The HPLC separations were performed using a Dionex 3000 HPLC System unit (Thermo Electron, USA) using a HSS T3 column (2.1 × 150 mm; 2.5 μm; Waters, UK) with a linear gradient of the binary solvent system under similar HPLC conditions as described above. The data-dependent mass spectrometry experiments were controlled using the menu-driven software provided with the Xcalibur system (ver. 2.2 SP1.48; Thermo Scientific, USA). The compounds were identified through comparisons with the standard compounds using the observed retention time, ultraviolet spectra, and mass chromatogram.

The peak 3 at 20.1 min co-eluted with the *trans*-piceid standard, and they had overlapping UV spectra with two absorbance maxima of 307 and 319 nm (Fig. 3 and 4A(b)). In contrast, a different UV spectrum with an absorbance maximum at 289 nm was obtained for the 24.7 min peak (Fig. 4A(c)). Under a positive ESI mode, the compound eluting at 20.1 min was generated at the *m/z* 390.92 $[M+H]^+$ ion. Its identity was confirmed as *trans*-piceid from the collision-induced dissociation (CID) spectrum (Fig. 4B(b)) of the *m/z* 390.92 $[M+H]^+$ ion with a prominent product ion at *m/z* 229.00 $[M+H]^+$ (protonated resveratrol), which indicates the loss of the glucose moiety. Interestingly, the compound eluting at 24.7 min exhibited a nearly identical molecular weight (*m/z* 390.96) and CID spectrum (Fig. 4B(c)). In order to identify these compounds unambiguously, *cis*-piceid standards were generated using the UV-induced isomerization of the *trans*-piceid standards [3]. The peak at 24.7 min was confirmed as *cis*-piceid. Interestingly, a similar molecular weight (*m/z* 391.08) was recorded for the compound eluting at 16.1 min, and it also produced a UV spectrum (Fig. 4A(a)) identical to that of the *trans*-piceid peak. The CID spectrum of the *m/z* 391.08 produced product ions at *m/z* 229.00 and *m/z* 211.08, which indicated the sequential loss of glucose and hydroxyl units. The presence of *m/z* 211.08 $[M+H]^+$ indicated that the two hydroxyl groups were located in the A-ring of resveratrol (Fig. S1). These data strongly suggest that peak 1 at 16.1 min is not glucoside formed on the A-ring but a glucoside on the B-ring of resveratrol (resveratrol 4′-*O*-glucoside;

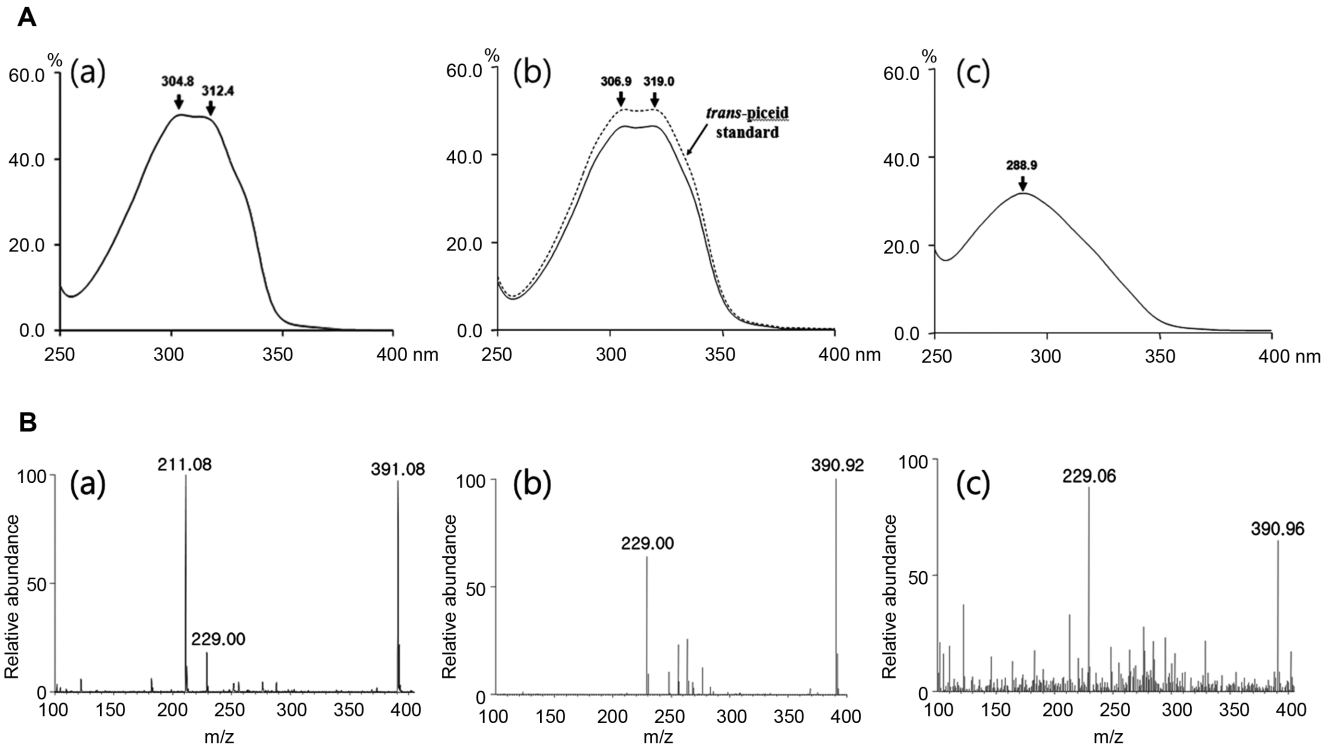

Fig. 4. (**A**) UV and (**B**) MS spectra for the distinct peaks ((a) 16.1, (b) 20.1 and (c) 24.7 min) detected in the engineered *E. coli*.
The dotted line in inset **A**(b) show the UV spectrum of the *trans*-piceid standard.

resveratroloside). However, the resveratrol 4′-*O*-glucoside cannot be confirmed as having a *trans*- or *cis*- form. Furthermore, the fermentation products of the *E. coli* cells harboring pET-opTLS demonstrated that *trans*-resveratrol was a major product. Thus, the product of *cis*-piceid in the *E. coli* cells harboring pET-opTLYS that is likely to result from the glucosylation step may facilitate the isomerization for long incubations in the culture medium. The amount of the *trans*-piceid was detected as 2.5 mg/l at the end of cultivation *via* the quantification of the corresponding standard. On the other hand, the *cis*-piceid and the resveratrol 4′-*O*-glucoside were roughly identified as 1.7 and 7.5 mg/l, respectively, *via* comparison with the spectral data interpretation values of the *trans*-piceid.

In conclusion, an *E. coli* system that contains an artificial biosynthetic pathway that produces resveratrol 3-*O*-glucoside and resveratrol 4′-*O*-glucoside from simple carbon source cultures was developed.

Acknowledgments

This work was supported in part by a grant from the KRIBB Research Initiative Program and Basic Science Research program (2012–0001421) funded by the NRF and by the Next-Generation BioGreen 21 Program (SSAC, PJ009549022013) funded by the RDA, Republic of Korea.

References

1. Ahn BC, Kim BG, Jeon YM, Lee EJ, Lim Y, Ahn J-H. 2009. Formation of flavone di-*O*-glucosides using a glycosyltransferase from *Bacillus cereus*. *J. Microbiol. Biotechnol.* **19:** 387-390.
2. Baur JA, Sinclair DA. 2006. Therapeutic potential of resveratrol: the *in vivo* evidence. *Nat. Rev. Drug Discov.* **5:** 493-506.
3. Camont L, Cottart CH, Rhayem Y, Nivet-Antoine V, Djelidi R, Collin F, *et al.* 2009. Simple spectrophotometric assessment of the *trans*-/*cis*-resveratrol ratio in aqueous solutions. *Anal. Chim. Acta* **634:** 121-128.
4. Choi O, Wu CZ, Kang SY, Ahn JS, Uhm TB, Hong Y-S. 2011. Biosynthesis of plant-specific phenylpropanoids by construction of an artificial biosynthetic pathway in *Escherichia coli*. *J. Ind. Microbiol. Biotechnol.* **38:** 1657-1665.
5. Cichewicz RH, Kouzi SA. 1998. Biotransformation of resveratrol to piceid by *Bacillus cereus*. *J. Nat. Prod.* **61:** 1313-1314.
6. Gurung RB, Kim EH, Oh TJ, Sohng JK. 2013. Enzymatic synthesis of apigenin glucosides by glucosyltransferase (YjiC) from *Bacillus licheniformis* DSM 13. *Mol Cells* **36:** 355-361.
7. Jang M, Cai L, Udeani GO, Slowing KV, Thomas CF, Beecher CW, *et al.* 1997. Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. *Science* **275:** 218-220.
8. Kaneko M, Ohnishi Y, Horinouchi S. 2003. Cinnamate:coenzyme A ligase from the filamentous bacterium *Streptomyces coelicolor* A3(2). *J. Bacteriol.* **185:** 20-27.
9. Kang SY, Choi O, Lee JK, Hwang BY, Uhm TB, Hong Y-S. 2012. Artificial biosynthesis of phenylpropanoic acids in a tyrosine overproducing *Escherichia coli* strain. *Microb. Cell Fact.* **11:** 153.
10. Khater F, Fournand D, Vialet S, Meudec E, Cheynier V, Terrier N. 2012. Identification and functional characterization of cDNAs coding for hydroxybenzoate/hydroxycinnamate glucosyltransferases co-expressed with genes related to proanthocyanidin biosynthesis. *J. Exp. Bot.* **63:** 1201-1214.
11. Ko JH, Kim BG, Ahn J-H. 2006. Glycosylation of flavonoids with a glycosyltransferase from *Bacillus cereus*. *FEMS Microbiol. Lett.* **258:** 263-268.
12. Ozaki S, Imai H, Iwakiri T, Sato T, Shimoda K, Nakayama T, Hamada H. 2012. Regioselective glucosidation of *trans*-resveratrol in *Escherichia coli* expressing glucosyltransferase from *Phytolacca americana*. *Biotechnol. Lett.* **34:** 475-481.
13. Pandey RP, Li TF, Kim EH, Yamaguchi T, Park YI, Kim JS, Sohng JK. 2013. Enzymatic synthesis of novel phloretin glucosides. *Appl. Environ. Microbiol.* **79:** 3516-3521.
14. Pandey RP, Parajuli P, Koirala N, Park JW, Sohng JK. 2013. Probing 3-hydroxyflavone for *in vitro* glycorandomization of flavonols by YjiC. *Appl. Environ. Microbiol.* **79:** 6833-6838.
15. Shim H, Hong W, Ahn BC. 2003. Enzymatic preparation of phenolic glucosides by *Streptococcus mutans*. *Bull. Kor. Chem. Soc.* **24:** 1680-1682.
16. Su D, Cheng Y, Liu M, Liu D, Cui H, Zhang B, *et al*. 2013. Comparision of piceid and resveratrol in antioxidation and antiproliferation activities *in vitro*. *PLoS One* **8:** e54505.
17. Torres P, Poveda A, Jimenez-Barbero J, Parra JL, Comelles F, Ballesteros AO, Plou FJ. 2011. Enzymatic synthesis of α-glucosides of resveratrol with surfactant activity. *Adv. Synth. Catal.* **353:** 1077-1086.
18. Walle T. 2011. Bioavailability of resveratrol. *Ann N. Y. Acad. Sci.* **1215:** 9-15.
19. Wang Y, Chen S, Yu O. 2011. Metabolic engineering of flavonoids in plants and microorganisms. *Appl. Microbiol. Biotechnol*. **91**: 949-956.
20. Watts KT, Lee PC, Schmidt-Dannert C. 2006. Biosynthesis of plant-specific stilbene polyketides in metabolically engineered *Escherichia coli*. *BMC Biotechnol.* **6**: 22.
21. Wu CZ, Jang JH, Woo M, Ahn JS, Kim JS, Hong Y-S. 2012. Enzymatic glycosylation of nonbenzoquinone geldanamycin analogs *via Bacillus* UDP-glycosyltransferase. *Appl. Environ. Microbiol.* **78:** 7680-7686.