Ann Microbiol (2015) 65:817–826
DOI 10.1007/s13213-014-0922-z

ORIGINAL ARTICLE

Pterostilbene production by microorganisms expressing resveratrol *O*-methyltransferase

Yechun Wang · Mohammad Wadud Bhuiya · Rui Zhou · Oliver Yu

Received: 28 February 2014 / Accepted: 26 May 2014 / Published online: 26 June 2014

Abstract Pterostilbene (3,5-dimethoxy-4′-hydroxyl-trans-stilbene)—a derivative of resveratrol—is a natural dietary compound and the primary antioxidant component in berries. Pterostilbene has significant advantages over resveratrol in bioavailability, half-life in the body, cellular uptake, oral absorption and metabolic stability. Here, we expressed the resveratrol *O*-methyltransferase (ROMT) gene (*VvROMT*) from grape (*Vitis vinifera*) in *Escherichia coli* and *Saccharomyces cerevisiae* and confirmed its specific ability to catalyze the production of pterostilbene from resveratrol. By co-expressing an additional two genes from the resveratrol biosynthetic pathway—4-coumarate CoA-ligase (*4CL*) and stilbene synthase (*STS*)—a large amount of pterostilbene was produced, with a trace amount of pinostilbene detected. To understand the molecular basis of the catalytic activity, four key amino acid residues were identified in a 3D-model of VvROMT and mutagenized and assayed for augmented catalytic activity. Our results demonstrate the potential utility of the engineered microorganisms for pterostilbene production and provide protein engineering targets that will hopefully lead to increased activity of the ROMT enzyme.

Keywords Pterostilbene · Resveratrol · Resveratrol *O*-methyltransferase · Metabolic engineering · Plant antioxidants

Electronic supplementary material The online version of this article (doi:10.1007/s13213-014-0922-z) contains supplementary material, which is available to authorized users.

Y. Wang (✉) · M. W. Bhuiya · R. Zhou · O. Yu
Conagen, 1005 North Warson Rd, Saint Louis, MO 63132, USA
e-mail: wangyechun320@gmail.com

O. Yu
Wuxi NewWay Biotech, 401 Xingyuan North Road, Wuxi, Jiangsu Province, China 214043

Introduction

Stilbenes comprise a small class of plant secondary metabolites derived from the phenylpropanoid pathway. The best-known stilbene is resveratrol (3,5,4′-trihydroxy-trans-stilebene). Resveratrol is a byproduct of plant response to outside stresses such as UV irradiation and fungal infection (Wang et al. 2010). Besides its important role as a phytoalexin in plant defense responses, resveratrol and its derivatives exhibit human health benefits, including anti-oxidant, anti-tumor and anti-aging activities (Jang et al. 1997; Campagna and Rivas 2010; Wang et al. 2010). One particularly promising derivative of resveratrol, pterostilbene, is found in grape (Schmidlin et al. 2008), indian kino tree (Paul et al. 1999) and blueberries (Rimando et al. 2004). Hundreds of studies have shown that pterostilbene associates with anti-cancer, anti-diabetic, anti-carcinogenic, anti-inflammatory and anti-lipogenic activities, as indicated for resveratrol (Rimando et al. 2002; Pari and Satheesh 2006; Remsberg et al. 2008; Lee et al. 2013). However, pterostilbene has several key advantages over resveratrol (Rimando et al. 2002; Paul et al. 2009; Fulda 2010), many of which can be attributed to the facts that pterostilbene contains two methoxy groups and one hydroxyl group while resveratrol has three hydroxyl groups. The two methoxy groups increase the hydrophobicity of pterostilbene, which increases oral absorption and enhances cellular uptake (Remsberg et al. 2008; Kapetanovic et al. 2011). Pterostilbene also has a longer half-life in the blood than resveratrol and has shown higher bioactivity in assays designed to test effectiveness in heart health and oxidative stress (Rimando et al. 2005; Joseph et al. 2008). Wilson et al. (2008) demonstrated that methoxylation products of resveratrol, including desoxyrhapontigenin, 3-hydroxy-5, 4′-dimethoxystilbene, pterostilbene, resveratrol-trimethylether but not pinostilbene, generally had increased bioactivity in the whole organism. Under their experimental conditions,

both hydroxylated and monomethoxylated stilbene, or pinostilbene, had negligible or modestly beneficial effects on adult *Caenorhabditis elegans* survival. Rimando et al. (2005) have shown that only pterostilbene is an agonist of peroxisome proliferator-activated receptor α (PPARα), but not resveratrol, piceatannol, or resveratrol trimethylether. Results from their in vitro study suggest that pterostilbene may be a more effective hypolipidemic agent than other forms of reservatrol.

Because both resveratrol and its methoxylated derivatives have various health benefits, metabolic engineering to increase their production has become an active field of research. Resveratrol production has been achieved in *Saccharomyces cerevisiae* and *Escherichia coli* (Becker et al. 2003; Beekwilder et al. 2006; Watts et al. 2006; Chemler and Koffas 2008; Trantas et al. 2009; Sydor et al. 2010; Wang et al. 2010, 2011; Lim et al. 2011; Jeandet et al. 2012; Wang and Yu 2012; Wu et al. 2013). However, reports on the production of methylated derivatives of resveratrol in microorganism and plants are few (Rimando et al. 2012; Jeong et al. 2014). Jeong et al. (2014) attempted to produce pterostilbene from resveratrol in *E. coli* by the expression of two putative genes encoding resveratrol *O*-methyltransferase (ROMT) from frost grape (*Vitis riparia, VrROMT*) and sorghum (*Sorghum bicolor, SbOMT*). Expression of *SbOMT* led to the production of pinostilbene from resveratrol, with only a trace amount of pterostilbene. Expression of *VrROMT* resulted in next to no methylated resveratrol (Jeong et al. 2014). Rimando et al. (2012) co-expressed *O*-methyltransferase of *Sorghum bicolor* (*SbOMT3*) and stilbene synthase of peanut (*AhSTS*) in tobacco and Arabidopsis, resulting in the accumulation of pterostilbene in both species.

We previously showed that yeast cells expressing a 4-coumarate CoA-ligase (*4CL*) and stilbene synthase (*STS*) fusion and gene produced 15-fold more resveratrol when fed 4-coumaric acid, the substrate of 4CL (Zhang et al. 2006). Resveratrol can be converted into pterostilbene and its methylated derivatives by expressing *ROMT* (Fig. 1). Many ROMTs have been identified and characterized from different species (Baerson et al. 2008; Schmidlin et al. 2008; Jeong et al. 2014). All the genes encoding enzymes responsible for pterostilbene biosynthesis have been cloned, making metabolic engineering of this compound relatively straightforward.

In the present study, we report the production of pterostilbene by co-expressing *4CL*, *STS* and *ROMT* in *E. coli* and *S. cerevisiae*. We showed that ROMT from grapevine (*Vitis vinifera*) was capable of yielding pterostilbene as the major product. We identified four key amino acid residues that contribute to substrate binding and affinity based on a three-dimensional (3D)-model structure of ROMT and site-directed mutagenesis.

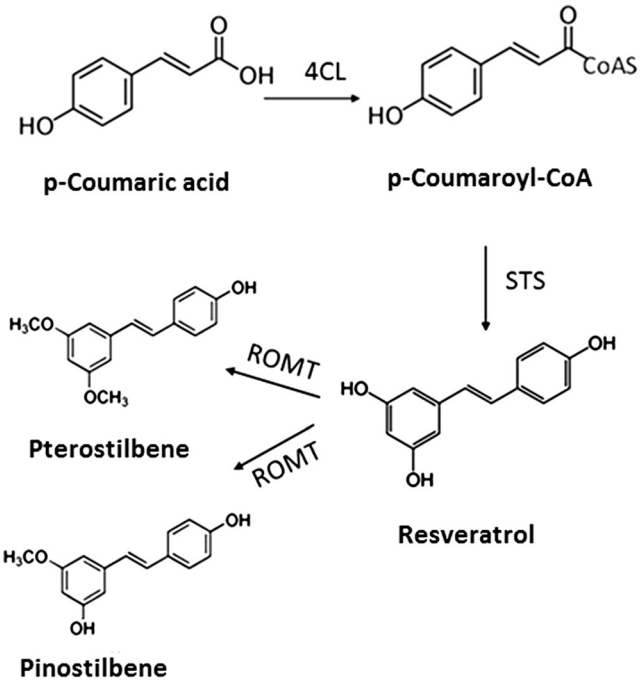


Fig. 1 Schematic diagram of the pterostilbene biosynthetic pathway. *4CL* 4-Coumarate:coenzyme A ligase, *STS* stilbene synthase, *ROMT* resveratrol *O*-methyltransferase

Materials and methods

Strains, plasmids and chemicals

Escherichia coli DH5α was used for plasmid cloning. *E. coli* BW27784 (Khlebnikov et al. 2001), kindly provided by Jay D. Keasling from the University of California, contains an integrated arabinose-H transporter gene, important for high-level resveratrol accumulation (Watts et al. 2006; Wang et al. 2011). Plasmids pAC-4CL1 (Watts et al. 2006) and pTrc-AtIPI (Cunningham et al. 2000) were kindly provided by Claudia Schmidt-Dannert from the University of Minnesota and Francis X. Cunningham Jr from the University of Maryland, respectively. *S. cerevisiae* strain WAT11 (Urban et al. 1997) was used as a host for gene expression. *p*-Coumaric acid, resveratrol and pterostilbene standards were purchased from Sigma (St. Louis, MO). Acetonitrile (HPLC grade) was purchased from EMD Chemicals (Gibbstown, NJ). Formic acid (HPLC grade) was purchased from Fisher Scientific (Pittsburgh, PA).

DNA manipulation

All DNA manipulations were performed according to standard procedures. Restriction enzymes and T4 DNA Ligase were purchased from New England Biolabs (Ipswich, MA). PCR amplification and cloning reactions were performed using Phusion® High-Fidelity DNA Polymerase (New England Biolabs). *Taq* DNA polymerase was used to add an A

to the blunt-end DNA fragments following the manufacturer's protocol (New England Biolabs).

RNA extraction and cDNA synthesis

ROMT (accession No: FM178870) was cloned from grape (*V. vinifera*). Grape total RNA was extracted with a Trizol Plus RNA Purification Kit (Life Technologies, Carlsbad, CA). The synthesis of cDNA was carried out with M-MuLV reverse transcriptase (New England Biolabs) following the manufacturer's manual. The genes were amplified from the synthesized cDNA with New England Biolabs' Phusion PCR Kit with the primers listed in Table 1.

Construction of expression vectors

ROMT was amplified with primers containing *Bam*HI and *Not*I restriction sites. The PCR product was first fractionated on agarose gel and then purified. The purified PCR fragment was digested by *Bam*HI and *Not*I, while plasmid pTrc-AtIPI (containing constitutive Trc promoter) was digested by *Bgl*II and *Not*I to remove the AtIPI gene. The *ROMT* was cloned into the empty pTrc vector to generate the plasmid pTrc-ROMT, which was verified by sequencing. To generate the SUMO-ROMT vectors for recombinant ROMT protein expression in *E. coli*, the ROMT gene was amplified by primers SumoROMTF and SumoROMTR. The amplified PCR product was cloned into pETite N-His SUMO vector (N-terminal His-tagged protein with SUMO solubility tag) according to the manufacturer's protocol (Lucigen, Middleton, WI). The resultant vectors were named SUMO-ROMT, SUMO-ROMT-167 (F167A), SUMO-ROMT-174 (D174A), SUMO-ROMT-258 (W258A) and SUMO-261 (H261A). Plasmid pAC-4CL1 was digested by *Xba*I and *Not*I. The vector pESC-TRP-4CL::STS (Zhang et al. 2006) was used as a template to amplify fusion gene *4CL::STS* with primers containing *Xba*I and *Not*I sites. The resulting 4CL::STS PCR product was cloned into the *Xba*I and *Not*I sites of pAC-4CL1 to generate pAC-4CLSTS. The ROMT gene was cloned into the Gateway entry vector using the pCR8/GW/TOPO TA Cloning kit (Life Technologies, Carlsbad, CA), was transformed into One Shot *E. coli* cells, and then sequenced. The ROMT gene was swapped into the Gateway destination vector pAG305GPD-ccdB (Addgene) by LR clonase II enzyme (Invitrogen). The resultant plasmid was named pAG305GPD-ROMT. Our previous plasmid, pAG304GPD-4CLSTS, was used to produce resveratrol (Wang et al. 2011). The yeast vectors contain an integrative recombination site and an expression cassette under the control of a constitutive promoter (GPD). These vectors were transformed into WAT11 for fermentation assays. Primers for all cloning reactions are available in Table 1.

Table 1 Primer pairs used in this study. *F* Forward, *R* reverse

Primer	Sequence (5′-3′)
RMOT-F	ATG GAT TTG GCA AAC GGT GTG ATA TC
RMOT-R	TCA AGG ATA AAC CTC AAT GAG GGA CC
SumoROMTF	CGC GAA CAG ATT GGA GGT GAT TTG GCA AAC GGT GTG ATA TCA GC
SumoROMTR	GTG GCG GCC GCT CTA TTA TCA AGG ATA AAC CTC AAT GAG GGA CC
4CL-F	ATG GCG CCA CAA GAA CAA GCA GTT TC
STS-R	TTA ATT TGT AAC CAT AGG AAT GCT ATG
4CL-XbaIF	GCT CTA GAA GGA GGA TTA CAA AAT GGC GCC ACA AGA ACA AGC AGT TTC TC
STS-NotIR	TTG CGG CCG CTT AAT TTG TAA CCA TAG GAA TGC TAT G
ROMT-BamHIF	CGG GAT CCA TGG ATT TGG CAA ACG GTG TGA TAT C
ROMT-NotIR	TTG CGG CCG CTC AAG GAT AAA CCT CAA TGA GGG ACC
Oligo dT (22)	TTT TTT TTT TTT TTT TTT TTV N
167F	GCT CAA CAA TTC CGC AAA TGA AGC CAT GGC
167R	GCC ATG GCT TCA TTT GCG GAA TTG TTG AGC
174F	GCC ATG GCT AGC GCA GCT CGC TTA CTC AC
174R	GTG AGT AAG CGA GCT GCG CTA GCC ATG GC
258F	CAA TTT TAC TCA AGG CAA TAC TGC ACG AC
258R	GTC GTG CAG TAT TGC CTT GAG TAA AAT TG
261F	CAA GTG GAT ACT GGC AGA CTG GAG CGA TG
261R	CAT CGC TCC AGT CTG CCA GTA TCC ACT TG

Cellular transformation

The yeast constructs pAG304GPD-4CLSTS and pAG305GPD-ROMT, along with pAG304GPD-ccdB and pAG305GPD-ccdB as controls, were transformed into WAT11 cells with the Frozen-EZ Yeast Transformation II kit (Zymo Research, Orange, CA). Vectors pAG304GPD-4CLSTS and pAG305GPD-ROMT were also co-transformed into WAT11 cells. The bacterial vectors pAC-4CLSTS and pTrc-ROMT were individually transformed and also co-transformed into *E. coli* BW27784.

Protein expression in recombinant *E. coli*

Escherichia coli BL21 (DE3) harboring the SUMO-ROMT and its four mutants constructs were grown in LB medium at 37 °C until OD_{600} reached 0.6. ROMT proteins were induced to express with 0.5 mM IPTG at 30 °C for 4 h. Cells were harvested by centrifugation (10,000 *g*; 5 min), re-suspended in the lysis buffer (50 mM Tris, pH 8.0, 10 mM imidazole, 500 mM NaCl, 10 % glycerol, and 1 mM EDTA), then disrupted by sonication (10 min, 30 s "on", 30 s "off"). The cell debris was clarified by centrifugation at 15,000 *g* for 5 min. The resultant supernatant was transferred to a fresh

tube, and the pellet was re-suspended in lysis buffer. Equal volumes from each fraction (soluble and insoluble) were mixed with SDS sample buffer and boiled at 100 °C for 4 min. An aliquote of each sample was analyzed by 10 % SDS-PAGE according to a standard protocol.

Production of resveratrol and pterostilbene in *E. coli*

A fresh, single colony of *E. coli* BW27784 strain harboring pAC-4CLSTS, pTrc-ROMT or both vectors was grown in 3 mL LB medium with either 34 μg/mL chloramphenicol, 100 μg/mL carbenicillin or both antibiotics overnight at 37 °C. From this starter culture, 500 μL was transferred to 50 mL M9 modified medium with antibiotics. M9 medium was modified by addition of yeast extract (1.25 g/L) and glycerol (0.5 % v/v) into standard M9 medium (Watts et al. 2006). *E. coli* BW27784 containing vectors was kept shaking at 200 rpm at 37 °C in modified M9 medium until OD_{600} reach 0.5, then *p*-coumaric acid (dissolved in 100 % ethanol) or resveratrol (dissolved in DMSO) was added to the culture to a final concentration of 0.328 g/L or 0.228 g/L, respectively. After a 4-day cultivation, samples (400 μL) were extracted with 800 μL ethyl acetate following the protocol of Wang et al. (2011). Extracts were evaporated to dryness with an Eppendorf Vacufuge (Eppendorf Scientific, Westbury, NY) at room temperature and re-dissolved in 80 % (v/v) methanol for HPLC analysis.

Production of resveratrol and pterostilbene in *S. cerevisiae*

For in vivo yeast assays, WAT11 cells containing pAG304GPD-4CLSTS, pAG305GPD-ROMT or both vectors were grown in standard yeast liquid drop-out medium at 30 °C with shaking (250 rpm) until the cell density reached OD_{600}= 0.2. The culture was then supplemented with either 10 mg/L *p*-coumaric acid every 24 h, based on our previous toxicity experiment (Wang et al. 2011), or 0.228 g/L resveratrol. The samples were collected at 4 days for resveratrol and pterostilbene analysis (Wang et al. 2011; Jeong et al. 2014). The metabolites were extracted with ethyl acetate using the above method. The entire experiment was repeated three times.

HPLC analysis

HPLC analysis of resveratrol and pterostilbene was carried out on a Dionex Ultimate 3000 system (http://www.dionex.com). Intermediates were separated by reverse-phase chromatography on a phenomenex Kinetex C18 column (particle size 2.6 μm; 150×4.6 mm; Phenomenex, Torrance, CA) with 0.1 % (v/v) formic acid (Solution A) and 100 % acetonitrile (Solution B). Samples were diluted into 80 % methanol. The following gradient procedure at a flow rate of 0.8 mL/min was used: 10 % of solution B for 2 min; a linear gradient from 10 to 70 % of solution B over 18 min; from 70 to 30 % of solution B over 1 min; from 30 to 10 % of solution B over 2 min; then 10 % of solution B for 5 min. For quantification, all intermediates were calibrated with external standards. The compounds were identified by their retention time as well as the corresponding UV spectrum, which were detected with a diode array in the system.

Homology modeling and docking for prediction of substrate binding residues of ROMT

To our knowledge, there is no tertiary structure of ROMT available that can be used for analysis of substrate binding sites. The 3D models of VvROMT and VrROMT were generated using the I-TASSER Protein Structure and Function Predictions web server (Roy et al. 2010). Homology modeling of ROMT was performed using the known 3D-structure of isoflavonoid O-methyltransferase (pdb code 1ZG3). The best model was ranked using the evaluation of TM-Score and RMSD. The best model of ROMT was confirmed based on TM-score (0.96), RMSD (1.6) and sequence identity (49.6 %) compared with pdb template. The 3D-model of ROMT dimer was built by superimposing the monomeric model of ROMT on stable dimer structure of 1ZG3 using the program COOT (Krissinel and Henrick 2004). The substrate binding site was predicted by docking resveratrol with the 3D-model of ROMT dimer using the computer program SwissDOCK (Grosdidier et al. 2011).

Site-directed mutagenesis of ROMT

Mutagenesis was performed at sites F167, D174, W258 and H261 of ROMT following the QuickChange site-directed mutagenesis strategy (Stratagene, La Jolla, CA). The primers used to construct mutants are listed in Table 1. The plasmid pTrc-ROMT was used as template. PCR was performed using Phusion high-fidelity DNA polymerase with the reaction settings at 98 °C for 30 s, followed by 25 cycles at 98 °C for 10 s, 60 °C for 20 s, and 72 °C for 3 min; the final extension was 72 °C for 7 min. The QuikChange PCR products were examined by agarose gel electrophoresis. To remove the template plasmid, 10 μL PCR product was digested with 1 μL *Dpn*I (New England Biolabs) at 37 °C overnight. An aliquot (2 μL) of digested products was transformed into BW27784 competent cells. Mutants were confirmed

by DNA sequencing of the pTrc-ROMT plasmids. The in vivo assays were as above.

Results

Expression of ROMT and its mutants in *E.coli*

Expression of recombinant ROMT and its four mutants was determined by SDS-PAGE. As shown in Fig. 2, the recombinant SUMO-ROMT and its four mutants showed a predicted molecular mass of about 52 kDa in soluble fraction. When total protein was separated into supernatant and pellet by centrifugation, SUMO-ROMOT appeared to be more abundant in the insoluble fraction than in the insoluble fraction (Fig. S1), probably due to the high induction temperature. A lower temperature may have increased the solubility of the recombinant protein (Weickert et al. 1996).

Conversion of resveratrol to pterostilbene via ROMT in recombinant *E. coli* and *S. cerevisiae*

To quantify the yield of products, four standards—*p*-coumaric acid, resveratrol, pinostilbene and pterostilbene—were analyzed by HPLC and shown to be well separated by the protocol (Fig. 3a). HPLC analysis showed that the BW27784 cells containing pTrc-ROMT produced three peaks. By comparison with retention times and UV spectra of the authentic standards, pterostilbene was identified from the cells expressing *ROMT* (Fig. 3). However, HPLC analysis indicated another small peak around 15.1 min (Fig. 3b, c), which has been identified as pinostilbene compared with the standard of pinostilbene, resulting from one methyl group being added onto resveratrol. Similar results were obtained in yeast (Fig. 3c). In *E. coli*, the efficiency of conversion was about 72 %, slightly higher than in *S. cerevisiae* (65 %). The cultured cells produced pterostilbene at concentrations of about 170±19 mg/L and 150±12 mg/L in *E. coli* and *S. cerevisiae*, respectively. Here, in both yeast and bacteria, pterostilbene was the major product and pinostilbene the minor product. Our result was consistent with a previous report of an in vitro test (Schmidlin et al. 2008). To our knowledge, this is the first report of pterostilbene as the major product in an engineered microorganism.

In a previous report, Jeong et al. (2014) showed that expression of *SbROMT3* in *E. coli* led to the production of pinostilbene (34 mg/L) from 1 mM resveratrol, with only trace amount of pterostilbene (0.16 mg/L) detected. Even lower amounts of pinostilbene and pterostilbene (0.16 mg/L and 0.04 mg/L, respectively) were detected in cells expressing *VrROMT*.

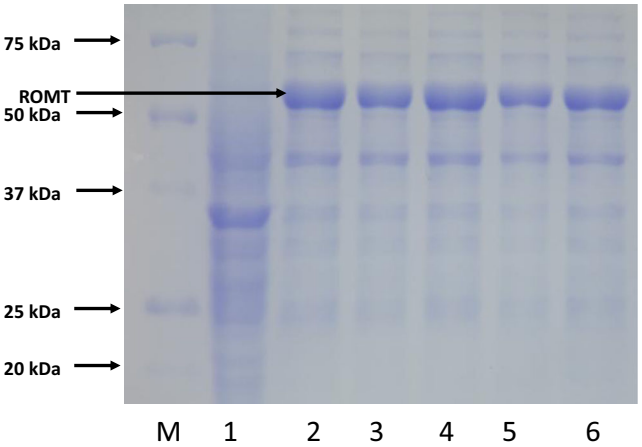


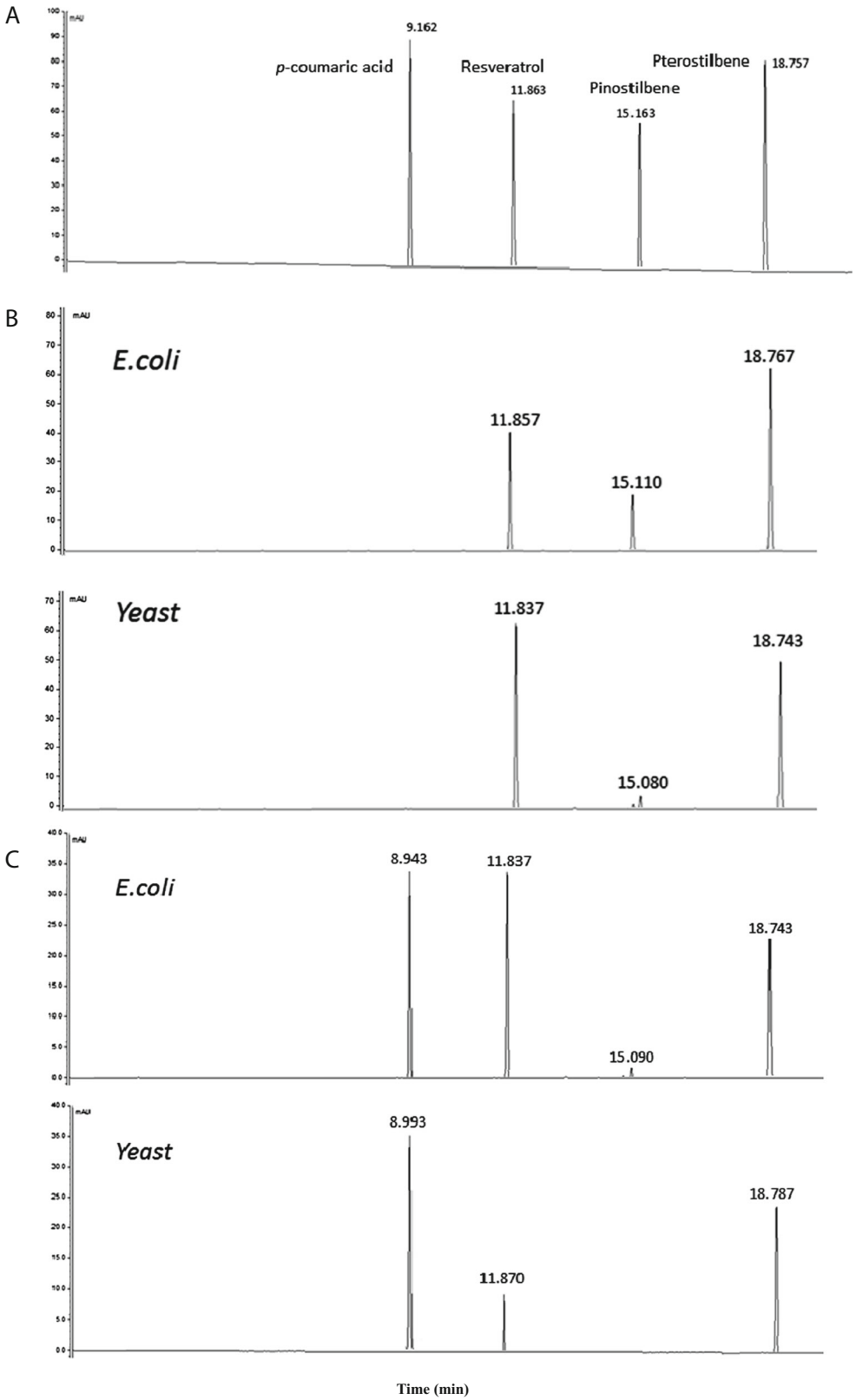
Fig. 2 Expression of the SUMO-tagged *Vitis vinifera* resveratrol *O*-methyltransferase gene (*VvROMT*) from grape and four mutant recombinant protein derivatives in *E. coli* BL21 (DE3) cells. *Escherichia coli* harboring either empty SUMO vector, SUMO-ROMT, SUMO-ROMT-167 (F167A), SUMO-ROMT-174 (D174A), SUMO-ROMT-258 (W258A) or SUMO-261 (H261A) vector were grown in LB medium and induced by the addition of 1 mM IPTG at 30 °C for 4 h. Proteins were separated by 10 % SDS-PAGE and stained with Coomassie brilliant blue. Lanes: *M* Protein molecular marker, *1* empty SUMO vector, *2* wild type SUMO-ROMT, *3* SUMO-ROMT-167 (F167A), *4* SUMO-ROMT-174 (D174A), *5* SUMO-ROMT-258 (W258A), *6* SUMO-261 (H261A)

Conversion of *p*-coumaric acid to pterostilbene via co-expression of *4CL::STS* and *ROMT* in recombinant *E. coli* and *S. cerevisiae*

Because *p*-coumaric acid is less expensive than resveratrol, we were motivated to test whether the strains co-expressing *4CL::STS* and *ROMT* can produce pterostilbene from *p*-coumaric acid directly. Using the strains developed here, *p*-coumaric acid could be converted into resveratrol in both BW27784 containing only pAC-4CLSTS and yeast harboring only pAG304GPD-4CLSTS in either modified M9 or yeast drop-out medium (date not shown). In the *E. coli* and *S. cerevisiae* cultures co-expressing *4CL::STS* and *ROMT*, *p*-coumaric acid was converted into both resveratrol and pterostilbene (Fig. 3c). In *E. coli* BW27784, when the cultures were fed *p*-coumaric acid directly, the yield of pterostilbene was about 50±8.2 mg/L (Fig. 3c); however, the pterostilbene yield was much lower (2.2±0.4 mg/L) in WAT11 cells (Fig. 3c). In both organisms, the resulting resveratrol was not converted completely into pterostilbene by ROMT (Fig. 3c), perhaps due to low activity of ROMT under the conditions used.

Prediction of substrate binding residues of ROMT based on the homology modeling

A 3D-model of ROMT was computed and analyzed structurally. After careful analysis of the substrate binding site, about 15 amino acid residues of ROMT were



predicted to be present in the binding pocket for the accommodated *S*-adenosyl methionine (SAM) and resveratrol in the putative active sites (Fig. 4). Four amino acid residues (F167, D174, W258 and H261) were predicted as potential catalytic amino acid residues for this enzyme activity (Zubieta et al. 2001). The amino acid residues

◀ **Fig. 3** **a**–**c** HPLC analysis of resveratrol, pinostilbene and pterostilbene produced by heterologous recombinant *E. coli* and *Saccharomyces cerevisiae*. **a** HPLC profiles of four authentic standards of *p*-coumaric acid, resveratrol, pinostilbene and pterostilbene with retention times of 9.162, 11.863, 15.163 and 18.757 min, respectively. **b** HPLC profiles of extracts from *E. coli* and *S. cerevisiae* cells expressing the *ROMT* gene. *E. coli* and *S. cerevisiae* cells containing pTrc-ROMT or pAG305GPD-ROMT were cultured as described in "Materials and methods". **c** HPLC profiles of extracts from *E. coli* and *S. cerevisiae* cells co-expressing the *4CL::STS* and *ROMT* genes. HPLC chromatograms showed the production of resveratrol and pterostilbene from *p*-coumaric acid in both microorganisms harboring either pAG304GPD-4CLSTS and pAG305GPD-ROMT or both plasmids pAC-4CLSTS and pTrc-ROMT

F167 and W258 form a sandwich to bind resveratrol; D174 is in close proximity to the substrate; and H261 might serve as a general base in the deprotonation of hydroxyl groups. Site-directed mutagenesis was used to construct four mutants of ROMT in order to determine the effect of these residues on enzyme activity. Each of the four distinct amino acid residues was replaced by alanine (A), then the ROMT of wild-type and four mutants were transformed into BW27784 competent cells, until OD_{600} reached 0.5, then resveratrol was added to the culture to a final concentration of 0.228 g/L. HPLC analysis showed that the mutant D174A retained only 3 % activity compared with wild type ROMT, while the other three mutants (F167A, W258A and H261A) lost their activity completely (Fig. 5). These results suggested that the amino acid residues at sites F167, D174, W258 and H261 are important for substrate binding and catalytic activity, and identified key amino acids as targets for improving ROMT activity. Saturation mutagenesis of ROMT is underway at sites F167, D174 and W258.

Discussion

The phenols pterostilbene and resveratrol, both components of foods such as red wine, are at least partially responsible for the so-called "French Paradox" due to their potential health benefits, including anti-cancer, anti-inflammatory, cardiovascular, and anti-diabetes activities, energy endurance enhancement, and protection against Alzheimer's disease (AD) (Chang et al. 2012; Riche et al. 2013). Recently, a study demonstrated for the first time that pterostilbene may have the potential to boost the innate immune system by increasing CAMP gene expression (Guo et al. 2013). Studies have already reported that pterostilbene is superior to resveratrol for certain health conditions and that it has promise for improving cardiovascular health, glucose levels, and cognitive function (Pari

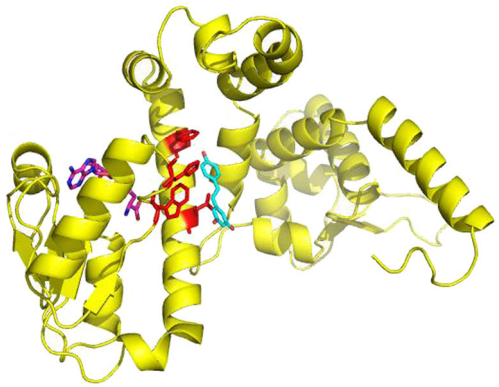


Fig. 4 Tertiary model of ROMT represented in *ribbon* form. Substrates are represented by *stick* model in *cyan*. Substrate binding residues are represented by stick model in *red*. F167A, D174A, W258A, H261A (predicted to be key amino acids for activity, with H261 being the most important) were constructed by site-directed mutagenesis

and Satheesh 2006; Joseph et al. 2008; Park et al. 2010). For example, compared to resveratrol, pterostilbene has a 7-fold longer half-life in the body (105 min vs 14 min) (Asensi et al. 2002; Remsberg et al. 2008), and 2–4 times greater cellular uptake, a reduced rate of elimination from the body (Lin et al. 2009; Kapetanovic et al. 2011), and higher oral bioavailability (80 % vs 20 %) (Kapetanovic et al. 2011). Chang et al. (2012) first demonstrated that pterostilbene is a more potent effector of beneficial molecular and functional events than resveratrol in SAMP8 mice, which have many of the histopathologic and behavior markers of AD including cognitive decline. Their study also showed that dietary supplementation of pterostilbene or resveratrol improved cognitive function in the SAMP8 mice and that pterostilbene showed significant improvement over resveratrol. In fact, even earlier—in 2006—Wen and Walle (2006) reported that methylated flavonoids appeared to demonstrate greatly improved intestinal absorption and metabolic stability. An additional methoxy group makes the compounds more hydrophobic, potentially leading to easier uptake through the cellular lipid bi-layer, which could explain the better bioavailablitity of pterostilbene and the more potent neuroprotective effect in the brain. Pterostilbene is generally safe for use in humans up to 250 mg/day and is well-tolerated at a twice daily dosing frequency (Riche et al. 2013).

On 7 June 2011, the FDA approved generally recognized as safe (GRAS) status for pterostilbene as a food ingredient (http://www.pteropure.com), which provides new commercial opportunities in natural food and beverage processing. However, as previously reported, pterostilbene production generally remains very low and inconstant, reported at concentrations of <5 μg/g FW in berries (Adrian et al. 2000) and at levels of 99–520 ng/g DW in two berry species in the heath family, *Vaccinium ashei* and *V. stamineum* (Rimando et al. 2004).

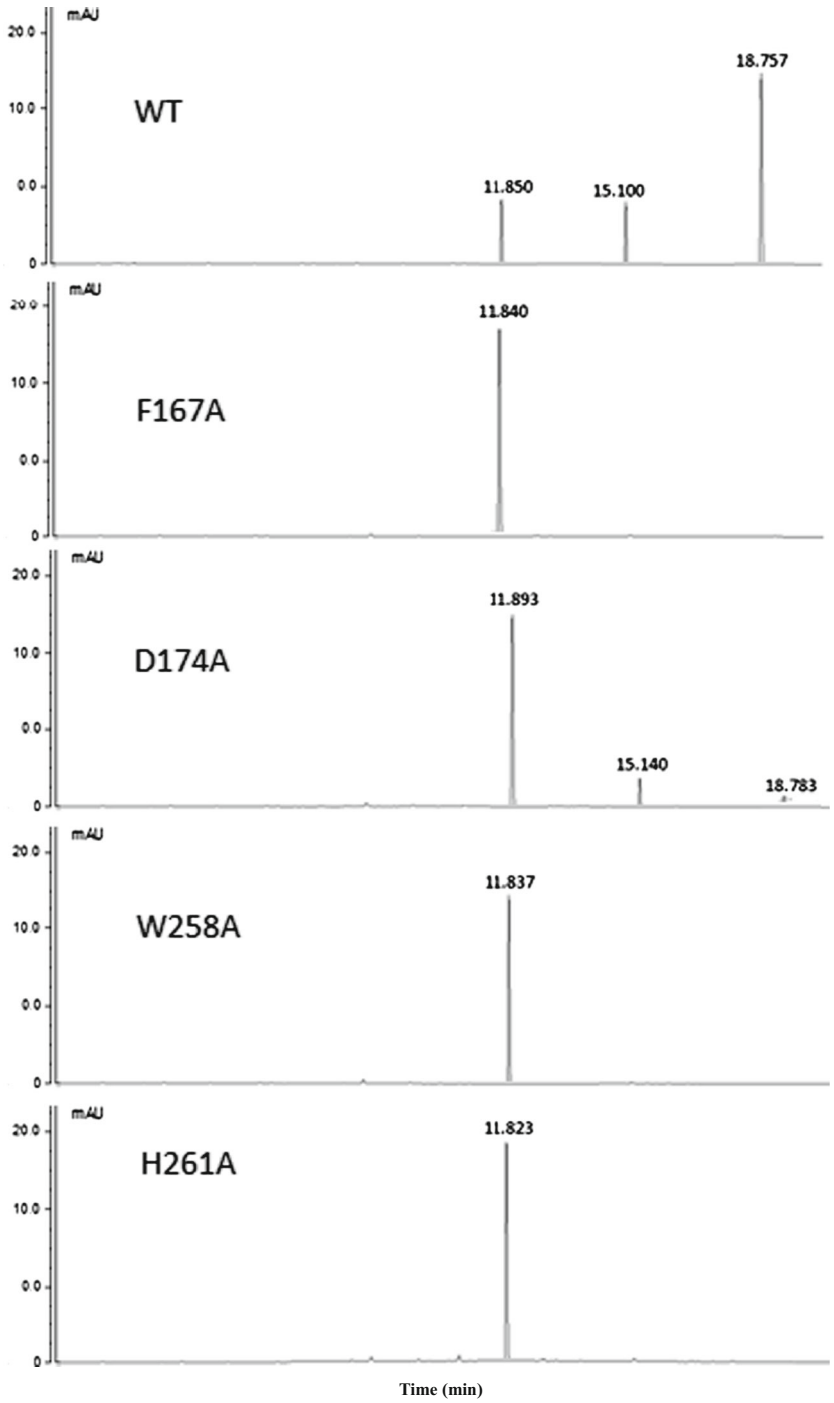


Fig. 5 HPLC analysis after expression in *E. coli* of *ROMT* or its four putative active site mutants. Chromatograms: *WT* wild-type *ROMT* gene; *F167A*, *D174A*, *W258A* and *H261A* showing products from the four mutated genes, respectively

Pterostilbene content is almost not detectable in leaves of *Vitis vinifera* cv *Cabernet Sauvignon*, reported at <50 μg/g DW in leaves infected with *Phomopsis viticola* (Schmidlin et al. 2008). Thus, engineering microorganisms to produce pterostilbene will have significant commercial value. There has been only one report on the production of pterostilbene in *E. coli* by expressing *VrROMT* and feeding resveratrol (Jeong et al. 2014). However, these bacteria yielded the major product pinostilbene (34 mg/L) and only low amounts of pterostilbene (0.16 mg/L). Wilson et al. (2008) reported that methoxylation of resveratrol increased bioactivity in *C. elegans* in vivo, but pinostilbene did not work. Therefore, pterostilbene might have higher commercial value than pinostilbene and even resveratrol.

The price of pterostilbene (US $700/kg) is higher than that of resveratrol (US $400/kg) and *p*-coumaric acid (US $50/kg) (personal communication, Steven Chen,

Blue California). There are no reports on the production of pterostilbene in microorganisms by feeding the less expensive substrate *p*-coumaric acid; therefore, engineering of pterostilbene biosynthesis from this precursor in microorganisms has significant scientific and economical importance. Several *O*-methyltransferases with substrates structurally analogous to resveratrol have been isolated from different species, including *Pinus sylvestris* (Chiron et al. 2000), *Rosa* spp. (Lavid et al. 2002), *Lolium perenne* (Tu et al. 2010) and *Solanum lycopersicum* (Mageroy et al. 2012). However, only four resveratrol-specific *O*-methyltransferases have been cloned and characterized, from *Vitis vinifera* (*VvROMT*) (Schmidlin et al. 2008), *Vitis riparia* (*VrROMT*) (Jeong et al. 2014) and *Sorghum bicolor* (*SbOMT1* and *SbOMT3*) (Baerson et al. 2008; Rimando et al. 2012). VvROMT exhibited lower K_m values and significantly higher K_{cat} and K_{cat}/K_m than SbOMT1 and SbOMT3. Taking advantage of these data, we attempted to use *E. coli* and *S. cerevisiae* to produce pterostilbene both by expressing *VvROMT* with feeding resveratrol and by co-expressing *4CL*, *STS* and *VvROMT* with feeding *p*-coumaric acid. In this initial engineering, 170±19 mg/L and 50±12 mg/L pterostilbene were produced from resveratrol and *p*-coumaric acid in *E. coli* BW27784, respectively. Pterostilbene was the major product and pinostilbene was the minor product in both organisms (Fig. 3b,c). Though VrROMT has 98 % sequence identity with VvROMT, they showed differences in their substrate specificity. The amino acid residues at sites 36, 92, 149, 152, 183, 187, 305 and 316 of VrROMT are different from the corresponding positions in VvROMT (Fig. S2). Structural analyses of these models showed that residues (Ile and Thr in VvROMT) at sites 305 and 316 at the edge of the loop in catalytic domain might contribute to their substrate specificity (Fig. S2, S3). Codon-optimization of *ROMT* for *S. cerevisiae* did not result in a significant increase (data not shown). Furthermore, site-directed mutagenesis of *ROMT* gave insights into improvements of pterostilbene production. Structure-guided saturation mutagenesis is in progress to further improve pterostilbene production. Besides site saturation mutagenesis, some other biotechnological methods, such as random mutagenesis, gene shuffling, engineering of the cell membrane and fermentation conditions optimization, etc., have been used to improve the efficiency of bioconversion.

In conclusion, we have provided the proof-of-concept for producing pterostilbene by co-expressing three genes, *At4CL*, *VvSTS* and *VvROMT*, to biosynthesize pterostilbene from *p*-coumaric acid in both *E. coli* and *S. cerevisiae*. Although the amount of pterostilbene was somewhat low, we believe that pterostilbene production will be further improved by increasing catalytic activity of ROMT by saturation mutagenesis of three key amino acids. Protein engineering of ROMT and optimizing metabolic engineering are the future direction of commercial production of pterostilbene.

Acknowledgments This work was supported by the National High Technology Research and Development Program of China (863 Program, 2013AA102801-03).

References

Adrian M, Jeandet P, Douillet-Breuil AC, Tesson L, Bessis R (2000) Stilbene content of mature *Vitis vinifera* berries in response to UV-C elicitation. J Agric Food Chem 48:6103–6105

Asensi M, Medina I, Ortega A, Carretero J, Bano MC, Obrador E, Estrela JM (2002) Inhibition of cancer growth by resveratrol is related to its low bioavailability. Free Radic Biol Med 33:387–398

Baerson SR, Dayan FE, Rimando AM, Nanayakkara NP, Liu CJ, Schroder J, Fishbein M, Pan Z, Kagan IA, Pratt LH, Cordonnier-Pratt MM, Duke SO (2008) A functional genomics investigation of allelochemical biosynthesis in *Sorghum bicolor* root hairs. J Biol Chem 283:3231–3247

Becker JV, Armstrong GO, van der Merwe MJ, Lambrechts MG, Vivier MA, Pretorius IS (2003) Metabolic engineering of *Saccharomyces cerevisiae* for the synthesis of the wine-related antioxidant resveratrol. FEMS Yeast Res 4:79–85

Beekwilder J, Wolswinkel R, Jonker H, Hall R, de Vos CH, Bovy A (2006) Production of resveratrol in recombinant microorganisms. Appl Environ Microbiol 72:5670–5672

Campagna M, Rivas C (2010) Antiviral activity of resveratrol. Biochem Soc Trans 38:50–53

Chang J, Rimando A, Pallas M, Camins A, Porquet D, Reeves J, Shukitt-Hale B, Smith MA, Joseph JA, Casadesus G (2012) Low-dose pterostilbene, but not resveratrol, is a potent neuromodulator in aging and Alzheimer's disease. Neurobiol Aging 33:2062–2071

Chemler JA, Koffas MA (2008) Metabolic engineering for plant natural product biosynthesis in microbes. Curr Opin Biotechnol 19:597–605

Chiron H, Drouet A, Lieutier F, Payer HD, Ernst D, Sandermann H Jr (2000) Gene induction of stilbene biosynthesis in Scots pine in response to ozone treatment, wounding, and fungal infection. Plant Physiol 124:865–872

Cunningham FX Jr, Lafond TP, Gantt E (2000) Evidence of a role for LytB in the nonmevalonate pathway of isoprenoid biosynthesis. J Bacteriol 182:5841–5848

Fulda S (2010) Resveratrol and derivatives for the prevention and treatment of cancer. Drug Discov Today 15:757–765

Grosdidier A, Zoete V, Michielin O (2011) SwissDock, a protein-small molecule docking web service based on EADock DSS. Nucleic Acids Res 39:W270–W277

Guo C, Sinnott B, Niu B, Lowry MB, Fantacone ML, Gombart AF (2013) Synergistic induction of human cathelicidin antimicrobial peptide gene expression by vitamin D and stilbenoids. Mol Nutr Food Res. doi:10.1002/mnfr.201300266

Jang M, Cai L, Udeani GO, Slowing KV, Thomas CF, Beecher CW, Fong HH, Farnsworth NR, Kinghorn AD, Mehta RG, Moon RC, Pezzuto JM (1997) Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. Science 275:218–220

Jeandet P, Delaunois B, Aziz A, Donnez D, Vasserot Y, Cordelier S, Courot E (2012) Metabolic engineering of yeast and plants for the production of the biologically active hydroxystilbene, resveratrol. J Biomed Biotechnol 2012:579089

Jeong YJ, An CH, Woo SG, Jeong HJ, Kim YM, Park SJ, Yoon BD, Kim CY (2014) Production of pinostilbene compounds by the expression of resveratrol *O*-methyltransferase genes in *Escherichia coli*. Enzym Microb Technol 54:8–14

Joseph JA, Fisher DR, Cheng V, Rimando AM, Shukitt-Hale B (2008) Cellular and behavioral effects of stilbene resveratrol analogues: implications for reducing the deleterious effects of aging. J Agric Food Chem 56:10544–10551

Kapetanovic IM, Muzzio M, Huang Z, Thompson TN, McCormick DL (2011) Pharmacokinetics, oral bioavailability, and metabolic profile of resveratrol and its dimethylether analog, pterostilbene, in rats. Cancer Chemother Pharmacol 68:593–601

Khlebnikov A, Datsenko KA, Skaug T, Wanner BL, Keasling JD (2001) Homogeneous expression of the P(BAD) promoter in *Escherichia coli* by constitutive expression of the low-affinity high-capacity AraE transporter. Microbiology 147:3241–3247

Krissinel E, Henrick K (2004) Secondary-structure matching (SSM), a new tool for fast protein structure alignment in three dimensions. Acta Crystallogr D60:2256–2268

Lavid N, Wang J, Shalit M, Guterman I, Bar E, Beuerle T, Menda N, Shafir S, Zamir D, Adam Z, Vainstein A, Weiss D, Pichersky E, Lewinsohn E (2002) *O*-methyltransferases involved in the biosynthesis of volatile phenolic derivatives in rose petals. Plant Physiol 129:1899–1907

Lee CM, Su YH, Huynh TT, Lee WH, Chiou JF, Lin YK, Hsiao M, Wu CH, Lin YF, Wu AT, Yeh CT (2013) Blue berry isolate, pterostilbene, functions as a potential anticancer stem cell agent in suppressing irradiation-mediated enrichment of hepatoma stem cells. Evid Based Complement Alternat Med 2013:258425

Lim CG, Fowler ZL, Hueller T, Schaffer S, Koffas MA (2011) High-yield resveratrol production in engineered *Escherichia coli*. Appl Environ Microbiol 77:3451–3460

Lin HS, Yue BD, Ho PC (2009) Determination of pterostilbene in rat plasma by a simple HPLC-UV method and its application in pre-clinical pharmacokinetic study. Biomed Chromatogr 23: 1308–1315

Mageroy MH, Tieman DM, Floystad A, Taylor MG, Klee HJ (2012) A *Solanum lycopersicum* catechol-O-methyltransferase involved in synthesis of the flavor molecule guaiacol. Plant J 69(6):1043–1051

Pari L, Satheesh MA (2006) Effect of pterostilbene on hepatic key enzymes of glucose metabolism in streptozotocin- and nicotinamide-induced diabetic rats. Life Sci 79:641–645

Park ES, Lim Y, Hong JT, Yoo HS, Lee CK, Pyo MY, Yun YP (2010) Pterostilbene, a natural dimethylated analog of resveratrol, inhibits rat aortic vascular smooth muscle cell proliferation by blocking Akt-dependent pathway. Vasc Pharmacol 53:61–67

Paul B, Masih I, Deopujari J, Charpentier C (1999) Occurrence of resveratrol and pterostilbene in age-old darakchasava, an ayurvedic medicine from India. J Ethnopharmacol 68:71–76

Paul S, Rimando AM, Lee HJ, Ji Y, Reddy BS, Suh N (2009) Anti-inflammatory action of pterostilbene is mediated through the p38 mitogen-activated protein kinase pathway in colon cancer cells. Cancer Prev Res (Phila) 2:650–657

Remsberg CM, Yanez JA, Ohgami Y, Vega-Villa KR, Rimando AM, Davies NM (2008) Pharmacometrics of pterostilbene: preclinical pharmacokinetics and metabolism, anticancer, antiinflammatory, antioxidant and analgesic activity. Phytother Res 22:169–179

Riche DM, McEwen CL, Riche KD, Sherman JJ, Wofford MR, Deschamp D, Griswold M (2013) Analysis of safety from a human clinical trial with pterostilbene. J Toxicol 2013:463595

Rimando AM, Cuendet M, Desmarchelier C, Mehta RG, Pezzuto JM, Duke SO (2002) Cancer chemopreventive and antioxidant activities of pterostilbene, a naturally occurring analogue of resveratrol. J Agric Food Chem 50:3453–3457

Rimando AM, Kalt W, Magee JB, Dewey J, Ballington JR (2004) Resveratrol, pterostilbene, and piceatannol in *Vaccinium* berries. J Agric Food Chem 52:4713–4719

Rimando AM, Nagmani R, Feller DR, Yokoyama W (2005) Pterostilbene, a new agonist for the peroxisome proliferator-activated receptor alpha-isoform, lowers plasma lipoproteins and cholesterol in hypercholesterolemic hamsters. J Agric Food Chem 53:3403–3407

Rimando AM, Pan Z, Polashock JJ, Dayan FE, Mizuno CS, Snook ME, Liu CJ, Baerson SR (2012) In planta production of the highly potent resveratrol analogue pterostilbene via stilbene synthase and O-methyltransferase co-expression. Plant Biotechnol J 10:269–283

Roy A, Kucukural A, Zhang Y (2010) I-TASSER: a unified platform for automated protein structure and function prediction. Nat Protoc 5: 725–738

Schmidlin L, Poutaraud A, Claudel P, Mestre P, Prado E, Santos-Rosa M, Wiedemann-Merdinoglu S, Karst F, Merdinoglu D, Hugueney P (2008) A stress-inducible resveratrol *O*-methyltransferase involved in the biosynthesis of pterostilbene in grapevine. Plant Physiol 148: 1630–1639

Sydor T, Schaffer S, Boles E (2010) Considerable increase in resveratrol production by recombinant industrial yeast strains with use of rich medium. Appl Environ Microbiol 76:3361–3363

Trantas E, Panopoulos N, Ververidis F (2009) Metabolic engineering of the complete pathway leading to heterologous biosynthesis of various flavonoids and stilbenoids in *Saccharomyces cerevisiae*. Metab Eng 11:355–366

Tu Y, Rochfort S, Liu Z, Ran Y, Griffith M, Badenhorst P, Louie GV, Bowman ME, Smith KF, Noel JP, Mouradov A, Spangenberg G (2010) Functional analyses of *caffeic acid O-Methyltransferase* and *Cinnamoyl-CoA-reductase* genes from perennial ryegrass (*Lolium perenne*). Plant Cell 22:3357–3373

Urban P, Mignotte C, Kazmaier M, Delorme F, Pompon D (1997) Cloning, yeast expression, and characterization of the coupling of two distantly related *Arabidopsis thaliana* NADPH-cytochrome P450 reductases with P450 CYP73A5. J Biol Chem 272:19176–19186

Wang Y, Yu O (2012) Synthetic scaffolds increased resveratrol biosynthesis in engineered yeast cells. J Biotechnol 157:258–260

Wang Y, Chen H, Yu O (2010) Metabolic engineering of resveratrol and other longevity boosting compounds. Biofactors 36:394–400

Wang Y, Halls C, Zhang J, Matsuno M, Zhang Y, Yu O (2011) Stepwise increase of resveratrol biosynthesis in yeast *Saccharomyces cerevisiae* by metabolic engineering. Metab Eng 13:455–463

Watts KT, Lee PC, Schmidt-Dannert C (2006) Biosynthesis of plant-specific stilbene polyketides in metabolically engineered *Escherichia coli*. BMC Biotechnol 6:22

Weickert MJ, Doherty DH, Best EA, Olins PO (1996) Optimization of heterologous protein production in *Escherichia coli*. Curr Opin Biotechnol 7:494–499

Wen X, Walle T (2006) Methylated flavonoids have greatly improved intestinal absorption and metabolic stability. Drug Metab Dispos 34: 1786–1792

Wilson MA, Rimando AM, Wolkow CA (2008) Methoxylation enhances stilbene bioactivity in *Caenorhabditis elegans*. BMC Pharmacol 8:15

Wu J, Liu P, Fan Y, Bao H, Du G, Zhou J, Chen J (2013) Multivariate modular metabolic engineering of *Escherichia coli* to produce resveratrol from L-tyrosine. J Biotechnol 167:404–411

Zhang Y, Li SZ, Li J, Pan X, Cahoon RE, Jaworski JG, Wang X, Jez JM, Chen F, Yu O (2006) Using unnatural protein fusions to engineer resveratrol biosynthesis in yeast and Mammalian cells. J Am Chem Soc 128:13030–13031

Zubieta C, He XZ, Dixon RA, Noel JP (2001) Structures of two natural product methyltransferases reveal the basis for substrate specificity in plant *O*-methyltransferases. Nat Struct Biol 8:271–279