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ORIGINAL PAPER

Construction, expression, and characterization of *Arabidopsis thaliana 4CL* and *Arachis hypogaea RS* fusion gene *4CL::RS* in *Escherichia coli*

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Abstract Resveratrol is an important antioxidant that confers several beneficial effects on human health. 4-coumarate coenzyme A ligase (*4CL*) and resveratrol synthase (*RS*) are key rate-limiting enzymes in the biosynthetic pathway of resveratrol. Using gene fusion technology, the fusion gene, *4CL::RS*, was constructed by the *4CL* gene from *Arabidopsis thaliana* and *RS* gene from *Arachis hypogaea*. DNAMAN analysis showed that the fusion gene encoded a 964-amino acid protein with an approximate weight of 104.7 kDa and a p*I* of 5.63. A prokaryotic expression vector containing *Nco*-I and *Eco*R-I restriction sites, pET-30a/*4CL::RS*, was identified by liquid culture bacterial PCR, enzyme digestion, and sequencing, and then used in the induction of expression. Subsequently, a biosynthetic pathway of resveratrol was constructed in *Escherichia coli* BL21(DE3) that harbored pET-30a/*4CL::RS*. The recombinant strains were induced to express the fusion protein at 28 °C for 8 h. After bacterial cells were disrupted by hypothermic ultrasonication, the *4CL::RS* fusion protein was thoroughly separated from tags using Ni–NTA affinity chromatography, and then detected by SDS-PAGE analysis. When the recombinant strains expressed the fusion protein, the precursor, *p*-coumaric acid, was converted to resveratrol. In the present study, the final concentration of resveratrol derived from 1 mM *p*-coumaric acid was 80.524 mg/L, with a 35.28 % (mol/mol) conversion yield.

Keywords Resveratrol · Fusion gene · Linker · *Arabidopsis thaliana* · *Arachis hypogaea*

Introduction

Resveratrol (3,5,4′-trihydroxy-*trans*-stilbene) is an important plant secondary metabolite of *Arachis hypogaea* and *Vitis vinifera* (Doré 2005). In the past few years, reports have described the beneficial effects of resveratrol on human health, which include anti-coagulant, anti-oxidation, anti-bacterial, anti-inflammatory, anti-cancer, cardiovascular protection, and neuroprotective properties (Sun et al. 2010; Athar et al. 2007; Li et al. 2012). Resveratrol has been detected in 72 plant species, representing 21 families and 31 genera (Burns et al. 2002). However, the content of resveratrol in plants is generally very low, making it difficult to meet market demands and in turn, rendering resveratrol biosynthesis as a highly interesting topic. The identification of a method of efficiently synthesizing resveratrol would not only address the problems of ecological destruction and resource scarcity associated with extracting resveratrol from plants, but also resolve problems relating to environmental pollution. The biosynthetic pathway of resveratrol involves the metabolic pathway of phenylpropanoid (Vogt 2010; Ferrer et al. 2008; Noel et al. 2005; Winkel 2004). A diagrammatic representation of the resveratrol pathway is presented in Fig. 1

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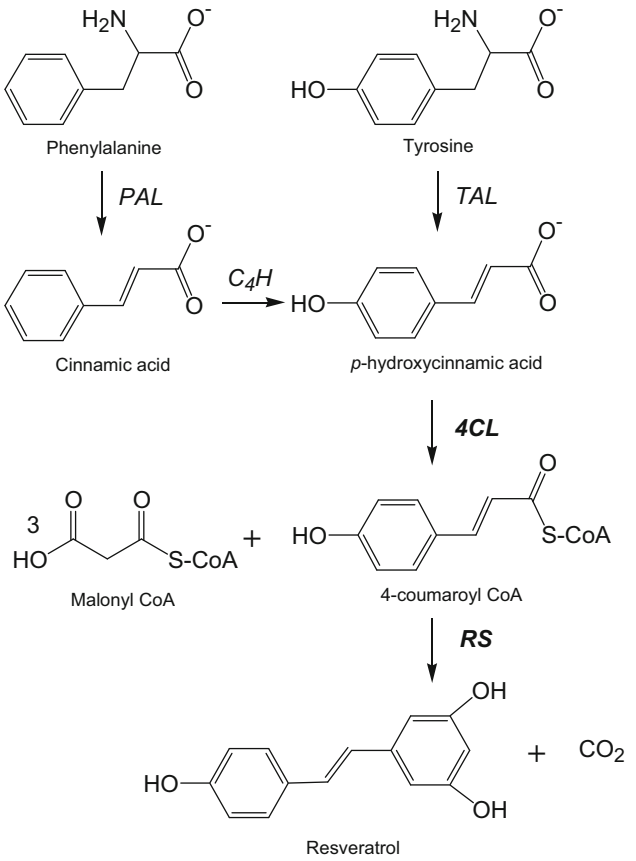

Fig. 1 Biosynthetic pathway of resveratrol. *PAL* phenylalanine ammonia lyase, *TAL* tyrosine ammonia lyase, *C_4H* cinnamate-4-hydroxylase, *4CL* 4-coumarate coenzyme A ligase, *RS* resveratrol synthase

(Donnez et al. 2009; Chong et al. 2009). In this pathway, the intermediate, *p*-coumaric acid, is generated through one-step and two-step enzymatic reactions involving phenylalanine and tyrosine, respectively (Wang et al. 2010). *p*-coumaric acid is ultimately converted into resveratrol through the catalyses of *4CL* and *RS*. To increase the production of resveratrol in microorganism, the *4CL* gene and *RS* gene were fused and integrated into a single expression box using gene fusion technology, thus resulting in the construction of a bifunctional enzyme. Based on these methods, the resveratrol was synthesized from the *p*-coumaric acid.

Bülow previously constructed bifunctional enzymes and analyzed multifunctional-enzyme molecules using a fusion gene (Bülow 1990; Bülow and Mosbach 1987). The fusion proteins not only retained enzyme activity but also imparted a detectable proximity effect on successive catalytic reactions. Studies have shown that when two or more different genes were fused, the activity of the fusion enzyme was directly influenced by the type and length of amino acids of the linker (Zhang et al. 2009). Because of the simple structure and various conformations of non-polar hydrophobic amino acids such as glycine (Gly, G) and serine (Ser, S), its potential application as the component of the linker was considered. The length of the amino acids in the linker ranged from 3 to 59 arbitrary units (A.U) (Robinson and Sauer 1998). For example, the extensive length of the linker renders the fusion protein to be easily biodegradable, whereas a very short linker induces instability due to higher-order structure folding and a low level of enzyme activity of the fusion proteins (Arai et al. 2001; Xue et al. 2004; LaVallie and McCoy 1995).

To construct the *4CL::RS* fusion gene in the present study, a 15-amino acid linker was inserted between the *4CL* gene from *Arabidopsis thaliana* and the *RS* gene from *A. hypogaea* using gene fusion technology. The *Bam*H-I restriction site was introduced in the linker, and then the *4CL* gene and *RS* gene were fused at this particular restriction site. A prokaryotic expression vector, pET-30a/*4CL::RS*, was constructed and then expressed. The fusion protein was isolated and purified using Ni–NTA affinity chromatography. Finally, the activity of the fusion protein was identified based on the quantity of the resveratrol derived from *p*-coumaric acid.

Materials and methods

Bacterial strains and plasmids

Escherichia coli TOP10 (Biomad Co., Ltd., Beijing, China) and *E. coli* BL21(DE3) (TransGen Biotech, Beijing, China) were used as host strains for cloning and expressing the fusion gene. The pMD19-T vector (TaKara, Tokyo, Japan) was used as cloning vector, whereas pET-30a(+) (Invitrogen) was used as expression vector. pMD18-T/*4CL* and pMD18-T/*RS* were kindly provided by Professor Xuefeng Guo of the International Centre for Bamboo and Rattan, China.

Design of the flexible linker

To increase total enzymatic efficiency, a 15-amino acid flexible linker was designed to connect the *4CL* gene and *RS* gene. It has a certain flexibility, which ensures the activity of the two enzymes. The sequences of the linker were designed as GGGGSGGGGSGGGGS. According to the sequences of the *4CL* gene and *RS* gene, as well as that of the *Bam*H-I restriction site, the nucleotide sequences of the flexible linker were designed as follows: ggtggaggcggatcc ggcggaggtggctct ggcggtggcggatcg (the underlined sequences represent the *Bam*H-I restriction site). The *Bam*H-I restriction site was introduced to facilitate the ligation of the *4CL* gene and *RS* gene.

Primer design

In the present study, primers were used to introduce the nucleotide sequences of the linker into the fusion gene. When the nucleotide sequences after the *Bam*H-I restriction site of the linker were placed in the forward primer of the second gene (*RS* gene), this primer would generate a fragment approximately 63 bp in size, which in turn would reduce its specificity. Therefore, this primer was designed as two parts. Five primers are listed in Table 1 using Primer Premier 5.0. Primers F_1 and R_1 were the upstream and downstream primers of the first gene (*4CL* gene), respectively. The *Nco*-I restriction site, CCATGG, was introduced into F_1. The *Bam*H-I restriction site was introduced into R_1, and the termination codon (TAG) of the *4CL* gene was removed. F_{21}, F_{22}, and R_2 were the primers of the *RS* gene. The *Bam*H-I restriction site was introduced into F_{22}, and the initiation codon (ATG) of the *RS* gene was eliminated. The *Eco*R-I restriction site, GAATTC, was introduced into R_2. Primers were synthesized and genes were sequenced by Beijing Biomad Co., Ltd.

Construction of the *4CL::RS* fusion gene

Gene fragment I, (*Nco*-I) CCA TGG + *4CL* (-TGA) + ggt gga ggc gga tcc (*Bam*H-I), was synthetized using pMD18-T/*4CL* as template, and F_1 and R_1 as primers.

The gene was amplified using pMD18-T/*RS* as a template and F_{21} and R_2 as primers, and then recovered. The gene fragment II, (*Bam*H-I) gga tcc ggc gga ggt ggc tct ggc ggt ggc gga tcg + *RS* (-ATG) + GAA TTC (*Eco*R-I), was synthesized with the recovered product as a template, and F_{22} and R_2 as primers.

After recovering gene fragments I and II, enzyme digestion of *Bam*H-I was conducted. The products were recovered using an extraction kit and ligated at 1:1 by the T_4 DNA ligase. The ligation efficiency was not high and the ligation product was not pure. Therefore, a PCR amplification experiment was performed using the final product of the ligation as template, and F_1 and R_2 as primers. The amplified fragment was recovered and ligated into a pMD19-T vector. The final plasmid, pMD19-T/*4CL::RS*, was transformed into *E. coli* TOP10. A single colony was selected and inoculated into the fresh medium and cultured for 12 h at 37 °C and 200 rpm, then sequenced.

Construction of pET-30a/*4CL::RS*

The plasmids extracted from the positive strains and pET-30a(+) were digested by *Nco*-I and *Eco*R-I, and then ligated by the T_4 DNA ligase. The recombinant plasmid, pET-30a/*4CL::RS*, was constructed and transfected into *E. coli* BL21(DE3) cells.

Expression of pET-30a/*4CL::RS*

Recombinant strains harboring pET-30a/*4CL::RS* were inoculated into liquid LB broth (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl) supplemented with kanamycin sulfate (30 mg/L) at a ratio of 1:50. After growing for 4 h (to an OD_{600} of approximately 0.5), the cultures were induced by adding 0.3 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside) and incubated for another 8 h at 28 °C. The bacterial cells expressing the desired enzyme were collected and lysed. Then inducible proteins were purified using Ni–NTA affinity chromatography and then detected using SDS-PAGE.

Measurement of *4CL::RS* bioactivity

Overnight cultures of recombinant strains with pET-30a/*4CL::RS* were inoculated at a ratio of 1:100 into 50 mL of LB containing kanamycin sulfate (30 mg/L) in 250 mL flasks at 37 °C and 200 rpm. When the OD_{600} reached 0.2, 1 mM *p*-coumaric acid in 200 μL DMSO and 0.3 mM IPTG were added to the cultures, which were then allowed to grow for an additional 48 h at 28 °C and 200 rpm. The blank control group was designed with strains harboring pET-30a and using the same procedure.

Table 1 Sequence of oligomers used in the present study

Oligomer No.	Oligomer sequence (5′ → 3′)
F_1	CATGCCATGGATGGCGCCACAAGAACAAGCAGTTTCT
R_1	CGCggatccgcctccaccCAATCCATTTGCTAGTTTTGC
F_{21}	ggctctggcggtggcggatcgGTGTCTGTGAGTGGAATT
F_{22}	CGCGGATCCggcggaggtggctctggcggtggcggatcg
R_2	CCGGAATTCTTATATGGCCATGCTGCGGAGGACAACAGT
T7 promoter	TAATACGACTCACTATAGGG
T7 terminator	TGCTAGTTATTGCTCAGCGG

The underlined sequences denote *Nco*-I, *Bam*H-I, *Bam*H-I and *Eco*R-I restriction sites, respectively. The lowercase characters indicate the nucleotide sequences of the linker

Sample preparation prior to HPLC (high performance liquid chromatography) analysis was performed as previously described (Watts et al. 2006). Briefly, 1 mL of the culture was centrifuged at 13,000 rpm for 5 min and the supernatant was transferred into a fresh 1.5 mL tube. Next, 50 μL of 1 M hydrochloric acid was added to the supernatant, and then the mixture was frozen overnight at −20 °C. Subsequently, the mixture solution was thawed out at room temperature and extracted three times with an equal volume (1 mL) of pure ethyl acetate. After removing the solvent with nitrogen gas, the dried residues were dissolved in 1 mL of methanol.

Resveratrol bioconversions were analyzed by HPLC (Waters 2695 separations module) by using a Zorbax SB-C18 column (5 μm particle size, 4.6 × 250 mm, Agilent Technologies, Palo Alto, CA, USA) and a Waters 2996 photodiode array detector (PDA). Resveratrol was eluted with an isocratic mobile phase consisting of 40 % methanol and 60 % water containing 0.1 % trifluoroacetic acid. The injection volume was 10 μL, the flow rate at the mobile phase was 1.0 mL/min, and chromatography was performed at 25 °C. The identification of resveratrol was conducted by comparing the retention time and UV spectrum against known standards. The standard curve of resveratrol was constructed by using six standard solutions (1, 5, 10, 50, 100, and 200 mg/L) (Sigma-Aldrich, Saint Louis, MO, USA) following the same procedure.

Results

Construction of the *4CL::RS* fusion gene

PCR products of gene fragments I and II were detected by agarose gel electrophoresis (Fig. 2). Agarose gel electrophoresis showed that gene fragment I was approximately 1700 bp in size. This was similar to the size of the *4CL* gene that lacked the termination codon, TGA, and contained the *Nco*-I restriction site, as well as the 15-bp section linker (containing the *Bam*H-I restriction site). On the other hand, gene fragment II was approximately 1200 bp in size, which was consistent the size of the *RS* gene lacking the initiation codon, ATG, and containing the other 30-bp section linker (also containing the *Bam*H-I restriction site), and the *Eco*R-I restriction site. The two gene fragments were recovered, and then ligated overnight after digestion with *Bam*H-I. Finally, the target gene was amplified with the final ligation product used as template, and the F_1 and R_2 as primers, and then detected by agarose gel electrophoresis (Fig. 2). The obtained fragment was 2913 bp in size, which was similar to that of the theoretical prediction of the *4CL::RS* sequence.

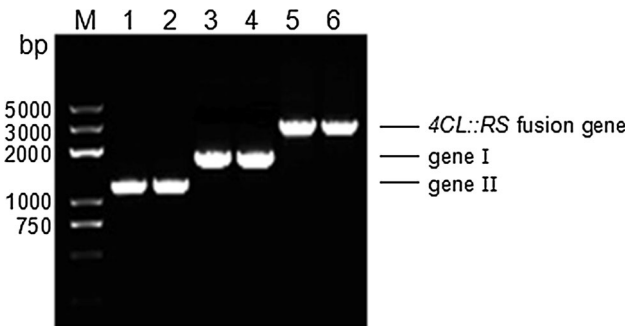

Fig. 2 PCR amplification of gene I, gene II, and the *4CL::RS* fusion gene. DNA molecular weight markers (Biomad Co., Ltd.) were used as DNA standard for the determination of molecular weights. *Lane M* BM5000 marker, *Lanes 1–2* gene I, *Lanes 3–4* gene II, *Lanes 5–6* *4CL::RS* fusion gene

Analysis of the *4CL::RS* fusion gene

Sequence analysis showed the full length of the *4CL::RS* fusion gene was 2895 bp, which included the *4CL* gene (1693 bp) without the termination codon, the *RS* gene (1167 bp) without the initiation codon, and the nucleotide sequence of the flexible linker (45 bp), which were similar to the details of the original design. The sequences of the fusion gene and the encoded amino acids were analyzed using DNAMAN (Fig. 3). Analyses showed that the fusion gene encoded for a 964-amino acid protein of approximately 104.7 kDa in size and a p*I* of 5.63.

Identification of pET-30a/*4CL::RS*

Liquid culture bacterial PCR

The single colony was inoculated into 1 mL of liquid LB containing kanamycin sulfate (30 mg/L) and cultured for 12 h at 37 °C and 200 rpm. In the present study, the negative control, pET-30a(+) was used as template, and the positive control, in which the final ligated product was used as template, were designed. The T7 primers were selected for liquid culture bacterial PCR (Table 1). The results were detected using agarose gel electrophoresis (Fig. 4). The amplified fragment of the negative control was 410 bp in size, which indicated that the nucleotide sequences of the forward and reverse primers of pET-30a(+) were 410 bp in size. The amplified fragment from liquid culture bacterial PCR was 3381 bp in size, which was similar to that observed in the positive control. This finding indicated that pET-30a/*4CL::RS* has been successfully constructed.

Restriction endonuclease digestion

The plasmids were extracted from positive strains that were verified by bacterial PCR, and treated with *Nco*-I, *Eco*R-I,

Fig. 3 Analysis of the *4CL::RS* fusion gene and its corresponding encoded amino acid sequences. ATG was the initiation codon of the *4CL::RS* fusion gene. A 15-amino-acid with frame served as the sequences of the linker. CCATGG and GAATTC indicated the *Nco*-I and *Eco*R-I restriction sites, respectively

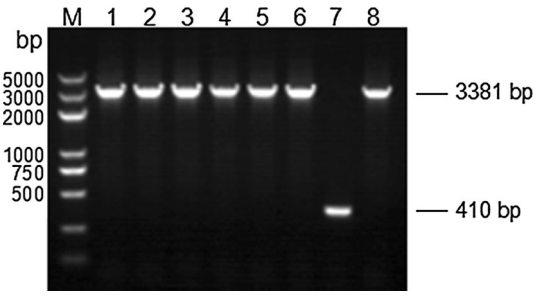

Fig. 4 Identification of liquid culture bacterial PCR for *E. coli* BL21 (DE3) cells transformed with pET-30a/*4CL::RS*. *Lane M* BM5000 marker, *Lanes 1–6* bacterial liquid, *Lane 7* negative control, *Lane 8* positive control

and double enzymes. The results were detected by agarose gel electrophoresis (Fig. 5). The recombinant plasmids after single-enzyme digestion were 8303 bp in size, whereas after double digestions were 5408 and 2895 bp in size, respectively. The product of the 5408 bp fragment was consistent with that observed when pET-30a(+) was digested with same enzymes, and the 2895 bp fragment was consistent with the size of the fusion gene fragment. The structure of the prokaryotic expression vector was therefore further verified.

Sequencing

Bacteria that had been identified as positive were sequenced. Sequence analyses showed that the *4CL::RS* fusion gene had been successfully ligated to the pET-30a by the *Nco*-I and *Eco*R-I restriction sites.

Induced expression of the fusion protein

The *4CL::RS* fusion protein was expressed by exogenously added IPTG and detected by SDS-PAGE (Fig. 6). Electrophoresis showed that the molecular weight of the expressed protein was approximately 105 kDa, which was consistent to its predicted molecular weight. The bacterial cells were collected and lysed by using hypothermic ultrasonication. The suspension was then centrifuged at 13,000 rpm for 30 min at 4 °C. The supernatant was purified using Ni–NTA affinity chromatography and detected by SDS-PAGE (Fig. 6). The results showed that fusion protein was purified after nickel affinity chromatography because the fusion protein contained a His-tag.

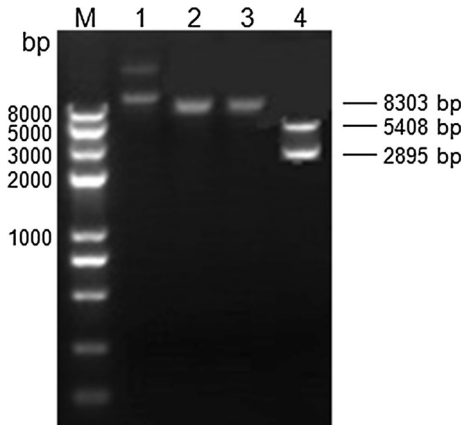

Fig. 5 Restriction enzyme digestion of pET-30a/*4CL::RS*. *Lane M* BM8000 marker, *Lane 1* no digestion of plasmid, *Lane 2 Nco*-I digestion of plasmid, *Lane 3 Eco*R-I digestion of plasmid, *Lane 4 Nco*-I and *Eco*R-I digestion of plasmid

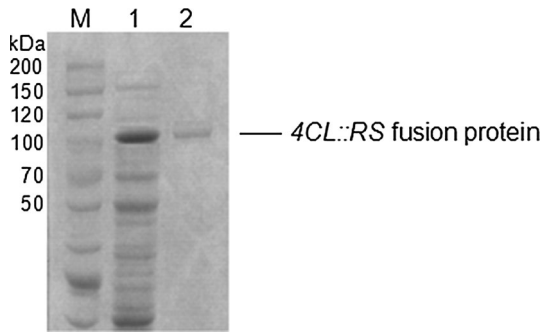

Fig. 6 SDS-PAGE analysis of the fusion protein. *4CL::RS* fusion proteins were obtained from *E. coli* BL21 (DE3) cells with or without Ni–NTA purification, and were analyzed on 8 % SDS-PAGE gels. Broad-range protein molecular weight markers (Biomad Co., Ltd.) were used as protein standard for the determination of molecular weights. *Lane M* protein marker, *Lane 1* total cell proteins of *E. coli* BL21 (DE3) containing pET-30a/*4CL::RS*, *Lane 2* purified protein

Assay of *4CL::RS* bioactivity

HPLC analyses of the product generated from recombinant strains with pET-30a/*4CL::RS* showed a peak with a retention time of 13 min and a UV spectrum of 305.7 nm, which were identical to that of authentic resveratrol (Fig. 7). Control cultures of *E. coli* containing pET-30a(+) did not show the same peak (data not shown). In the present study, the standard curve of resveratrol was calculated as follows:

$$Y = 25217 \times X - 25296;$$

wherein Y = peak area and X = compound concentration.

The correlation coefficient (R^2) of this regression equation was 0.9998. After 48 h of growth, 80.524 mg/L resveratrol was detected in the culture media of the experimental group with 1 mM *p*-coumaric acid, which was equivalent to a 35.28 % conversion yield (mol/mol). Taken together, the results indicated that the fusion protein expressed from the recombinant strain possessed the activities of the *4CL* and *RS* enzymes.

Discussion

A fusion gene was designed using a flexible peptide containing 15 neutral amino acids (G, S) that was inserted between the *RS* gene and the *4CL* gene. This flexible peptide is easy to bend and is elastic, and this property may facilitate the correct folding of the two proteins so that their original functions are not affected.

Previous studies on the microbial production of resveratrol showed that the *RS* gene of *A. hypogaea*, *V. vinifera*, *Pinus massoniana*, and *Pinus strobus* L. encodes an active

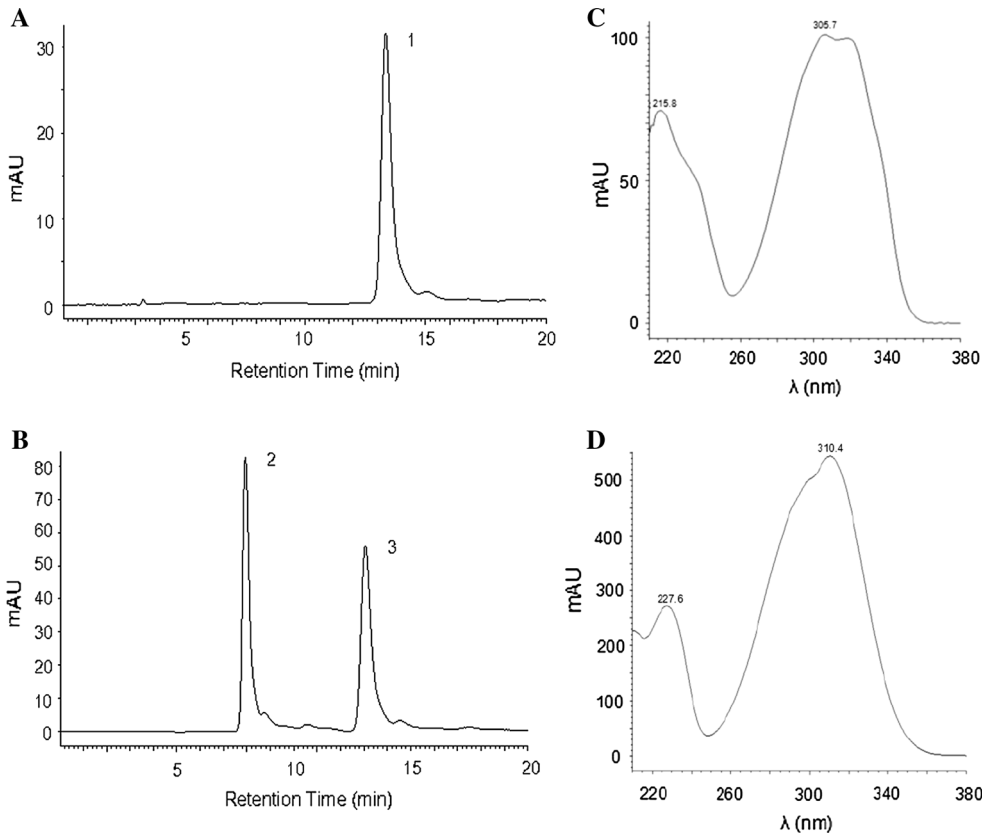

Fig. 7 HPLC analysis of resveratrol produced by recombinant *E. coli* BL21(DE3) cells with pET-30a/*4CL::RS*. **A** shows the chromatogram of authentic resveratrol (Sigma-Aldrich), and **B** represents the samples produced by recombinant strains expressing *4CL* and *RS* in LB media with *p*-coumaric acid in a shaking flask culture at 28 °C. The *peaks 1* and *3* at 13 min represent resveratrol, which was monitored using a PAD (photodiode array detector) at a wavelength of 305.7 nm (**C**). *Peak 2* at 8 min shows an absorption wavelength of 310.4 nm (**D**), representing *p*-coumaric acid

proteins in *E. coli*, and the activity of *RS* from *A. hypogaea* and *V. vinifera* was significantly higher than that observed in the other species (Lim et al. 2011). To increase resveratrol yield, a fusion protein, *4CL::RS*, was constructed using *4CL* from *A. thaliana* and *RS* from *V. vinifera*, which increased resveratrol production in yeast to up to 5.35 mg/L (32.11 % mol/mol) (Zhang et al. 2006). On the other hand, a recombinant plasmid, pUC-*Vvsts-At4cl1*, which co-expressed the *4CL* gene from *A. thaliana* and the *RS* gene from *V. vinifera*, yielded a final concentration of resveratrol of up to 2340 mg/L, with a 68.35 mol% conversion yield (Lim et al. 2011).

In the present study, the fusion gene was successfully constructed by the *Bam*H-I site and the fusion protein displayed the bioactivities of the both enzymes. This research results were different from the previous report (Watts et al. 2006). No studies on building a *4CL::RS* fusion gene using this method have been reported to date. Using our experimental conditionals, our recombinant strain produced 80.524 mg/L of resveratrol within 48 h. The present study provides baseline information that could be utilized in future studies on the optimization of resveratrol fermentation, which in turn could potentially increase the productivity of resveratrol.

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