

Full Length Research Paper

# Cloning and characterization of a $\gamma$ -tocopherol methyltransferase from Chinese cabbage

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Accepted 17 August, 2012

$\alpha$ -Tocopherol, with antioxidant properties, is synthesized by photosynthetic organisms and play important roles in human and animal nutrition. The final step of the  $\alpha$ -tocopherol biosynthetic pathway is catalyzed by  $\gamma$ -tocopherol methyltransferase ( $\gamma$ -TMT). In the major oilseed crops,  $\gamma$ -tocopherol, the biosynthetic precursor of  $\alpha$ -tocopherol, is the predominant form. The full-length cDNA of  $\gamma$ -TMT was obtained from Chinese cabbage (*Brassica. rapa*, Pekinensis Group), named BoTMT, by reverse transcription polymerase chain reaction (RT-PCR). Sequence analysis indicates that, BoTMT gene consisted of the open reading frame of 1041 nucleotides encoding a protein of 39 kD polypeptide. A BoTMT whole cell system was developed for the production of  $\alpha$ -tocopherol through the expression of BoTMT in *Escherichia coli* BL21 (DE3) strain.

**Key words:**  $\gamma$ -Tocopherol methyltransferase, Chinese cabbage, *Perilla frutescens*, tocopherol, high performance liquid chromatography (HPLC).

## INTRODUCTION

$\alpha$ -Tocopherol is considered as the most important form of vitamin E for human health, as it has ten-fold higher antioxidant activity than other tocopherols (Traber et al., 1996; Jiang et al., 2009). The bioavailability and bioactivity of  $\gamma$ -tocopherol are lower than those of  $\alpha$ -tocopherol (Traber et al., 1996; Jiang and Ames, 2003, 2009).  $\alpha$ -Tocopherol is known to be localized and synthesized in plastids (Munné-Bosch and Alegre, 2002), and enzymes involved in its biosynthesis are localized inside the plastid (Munné-Bosch and Alegre, 2002; Soll et al., 1985). The pathway for biosynthesis has been elucidated (Austin et al., 2006; Vidi et al., 2006; Ytterberg et al., 2006), including the phytylation of homogentisic acid to

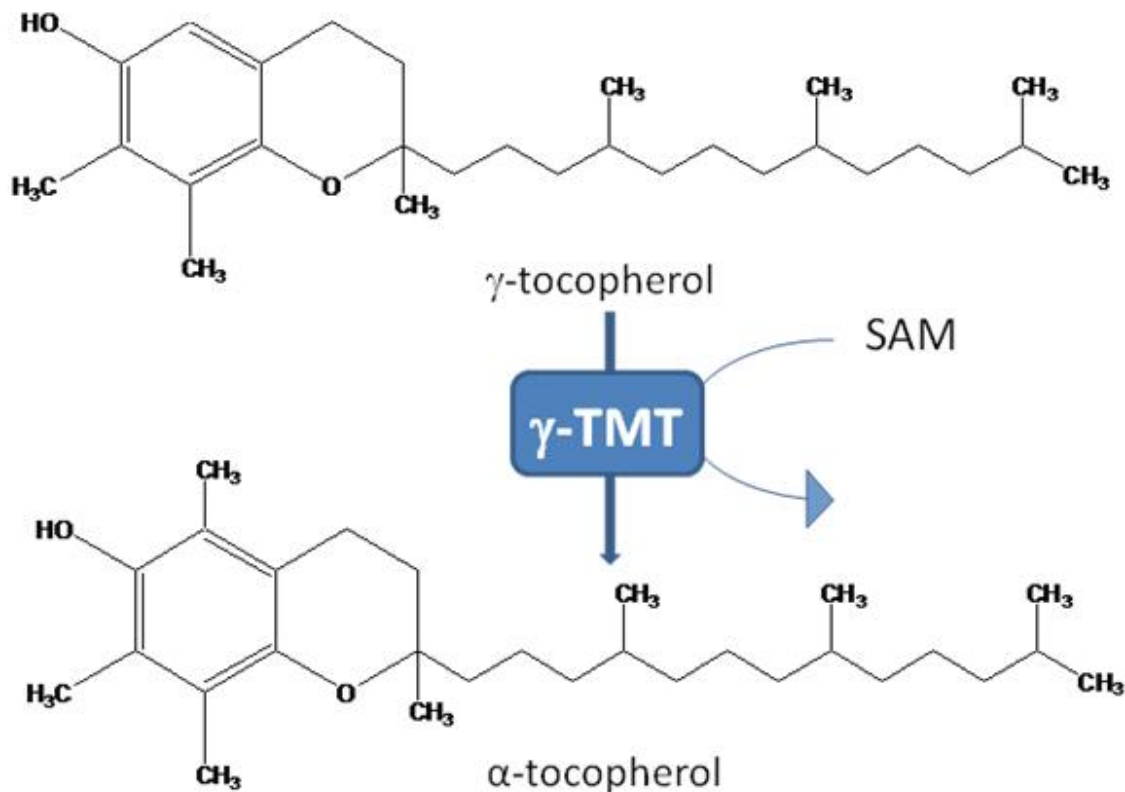
form 2-methyl-6-phytylquinol, the first ring methylation at position three to yield 2,3-dimethyl-5-phytylquinol, cyclization to yield  $\gamma$ -tocopherol, and finally a second ring methylation at position five to yield  $\alpha$ -tocopherol (Figure 1) by  $\gamma$ -tocopherol methyltransferase ( $\gamma$ -TMT).

Overexpression of *Perilla frutescens*  $\gamma$ -TMT alone increased  $\alpha$ -tocopherol to more than 90% of total tocopherol (Tavva et al., 2007).  $\gamma$ -TMT was identified from model organisms *Arabidopsis*, soybean and *Synechocystis* through genomics based approach (Shintani and Dellapenna, 1998; Grusak and Dellapenna, 1999; Koch et al., 2003; Dwiyananti et al., 2011). Overexpression of the  $\gamma$ -TMT of *Arabidopsis* with a seed-specific promoter resulted in a more than 80 fold increase of  $\alpha$ -tocopherol at the expense of  $\gamma$ -tocopherol (Koch et al., 2003). These researches suggest that the final enzyme  $\gamma$ -TMT of the tocopherol biosynthesis pathway is likely limited in the seeds of most agriculturally important crop (Jiang et al., 2009; Mohd and Neera, 2007).

For understanding the biochemical pathway of tocopherol biosynthesis and for improving the nutritional quality of crop plants, a full-length cDNA of  $\gamma$ -TMT was

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**Abbreviations:**  $\gamma$ -TMT,  $\gamma$ -Tocopherol methyltransferase; RT-PCR, reverse transcription polymerase chain reaction; HPLC, high performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis



**Figure 1.**  $\gamma$ -TMT enzymatic reaction.  $\gamma$ -TMT adds a methyl group to ring carbon 5 of  $\gamma$ -tocopherol.

obtained from Chinese cabbage (*Brassica rapa*, Pekinensis Group), named BoTMT in this study. We attempted a detailed characterization of BoTMT activity with expression in *Escherichia coli*.

## MATERIALS AND METHODS

### Plant materials, strains, plasmid and major reagent

Leaves of Chinese cabbage and *Perilla frutescens* were used to isolate total RNA. *E. coli* DH5 $\alpha$ , BL21(DE3), and plasmid pET28a were purchased from Merck. RNAiso for polysaccharide-rich plant Tissue, PrimeScript™ II 1st strand cDNA synthesis kit and the sequencing vector pMD19-T were from TaKaRa. DNA Gel extraction Min Kit was purchased from Axygen Company. Restriction endonuclease, T4 DNA ligase, and Taq DNA polymerase were from Fermentas. Polymerase chain reaction (PCR) primers were synthesized by Sangon (Shanghai, China).  $\gamma$ -,  $\alpha$ -tocopherols, S-adenosylmethionine (SAM) was purchased from Sigma. Chromatographic materials and columns were obtained from Agilent Technologies.

### Total RNA extraction, reverse transcriptase reactions, $\gamma$ -TMT cloning, primers and sequencing

Total RNA was extracted by using RNAiso™ for polysaccharide-rich plant tissue solution according to the manufacturer's directions.

Total RNA was used for 1st strand cDNA synthesis reaction by using PrimeScript™ II 1st strand cDNA synthesis kit according to the manufacturer's instructions. *BoTMT* gene was carried out with primers P1BoTMT/P2BoTMT. The primers used in this study were listed in Table 1.

### Construction of *E. coli* expression vectors

PCR amplifications of the full coding region BoTMT and *P. frutescens*  $\gamma$ -TMT were carried out with primers P3BoTMT/P4BoTMT and PfTMT/PfTMT, respectively. The cDNAs were used as templates.

The PCR products digested with *Bam*HI/*Sal*I, were inserted at the *Bam*HI/*Sal*I site of plasmid pET28a to generate express vectors pET28a-TMT-Bo and pET28a-TMT-Pf (Figures 2a and b), respectively.

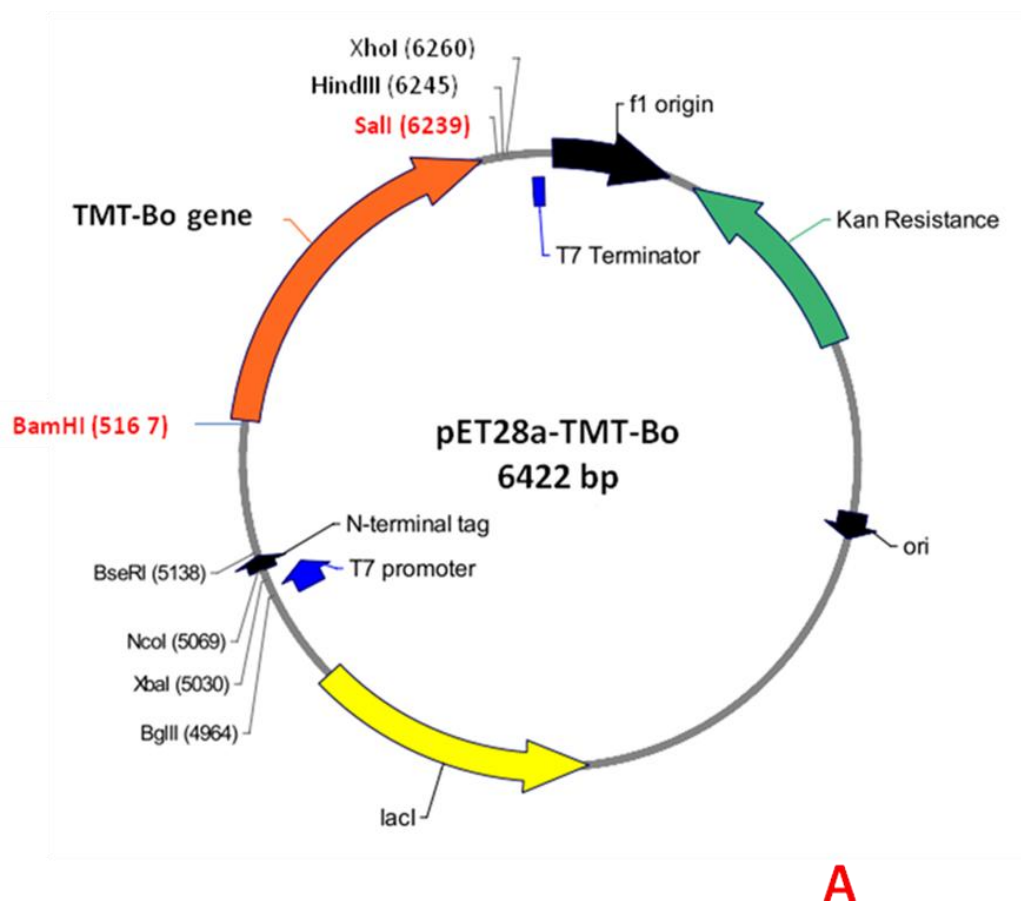
### Expression BoTMT and *P. frutescens* $\gamma$ -TMT in *E. coli* BL21

The transformants harboring plasmid pET28a-TMT-Bo and pET28a-TMT-Pf were cultured at 37°C in LB medium until OD<sub>600</sub> nm reached 0.5 to 0.7. Isopropylthio- $\beta$ -galactoside (IPTG) was added to the final concentration of 0.5 mmol/L and the cultivation was continued for another 4 to 5 h at 37°C. The cell was harvested by centrifugation. The protein concentration was measured by Bio-Rad Protein Assay. Total bacterial protein was analysed by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

**Table 1.** Primers used in this study.

| Primer               | Sequence (5'-3')  |
|----------------------|---|
| P1BoTMT              | ATGAAAGCGACTCTCGCAC                                     |
| P2BoTMT              | TTAGAGAGGCTTCTGGCAA                                     |
| P3BoTMT <sup>a</sup> | <u>GGATCC</u> (BamHI) <b>GATG</b> AAAGCGACTCTCGCAC      |
| P4BoTMT <sup>b</sup> | <u>GTCGAC</u> (SalI) <b>TTA</b> GAGAGGCTTCTGGCAA        |
| PfTMT <sup>a</sup>   | <u>GGATCC</u> (BamHI) <b>GATG</b> GCGGAGATGGAGACGGA     |
| PfTMT <sup>b</sup>   | <u>GTCGAC</u> (SalI) <b>TTA</b> AGATGCAGGTTTTTCGGCATGTA |

<sup>a</sup>The *Bam*HI restriction site is underlined, the start codon site is bold, <sup>b</sup>The *Sal*I restriction site is underlined, the stop codon site is bold.



**Figure 2.** Schematic diagram of the expression vectors. (a) pET28a-TMT-Bo (6422 bp), (b) pET28a-TMT-Pf (6269 bp).

### The enzyme activity assay of the recombinant $\gamma$ -TMT

*E. coli* cells carrying pET28a-TMT-Bo, pET28a-TMT-Pf and pET28a, respectively, were ground with liquid nitrogen and suspended in 100 mM  $K_2HPO_4$ - $KH_2PO_4$ , pH 6.5. Extracts were harvested by centrifugation and 100  $\mu$ l of supernatant were used for  $\gamma$ -TMT activity analysis. The  $\gamma$ -TMT activity assay was performed as described by Shitani (1998) except that the final concentrations of  $\gamma$ -tocopherol and SAM were 0.02 and 1 mmol/L, respectively. The enzyme activity was measured by assessing the residual enzyme

activity after incubated in shaking incubator (150 rpm) at 30°C for 3 h.

### Chemical analysis

The  $\gamma$ -,  $\alpha$ -tocopherol stock was prepared by dissolving 30 mg of  $\alpha$ -tocopherol in 100 ml of methanol-acetonitrile (30:70 v/v), giving a final concentration of 300  $\mu$ g/ml. The stock was used to obtain working solutions of 0.75, 1.5, 3.0 and 6.0  $\mu$ g/ml which were stored

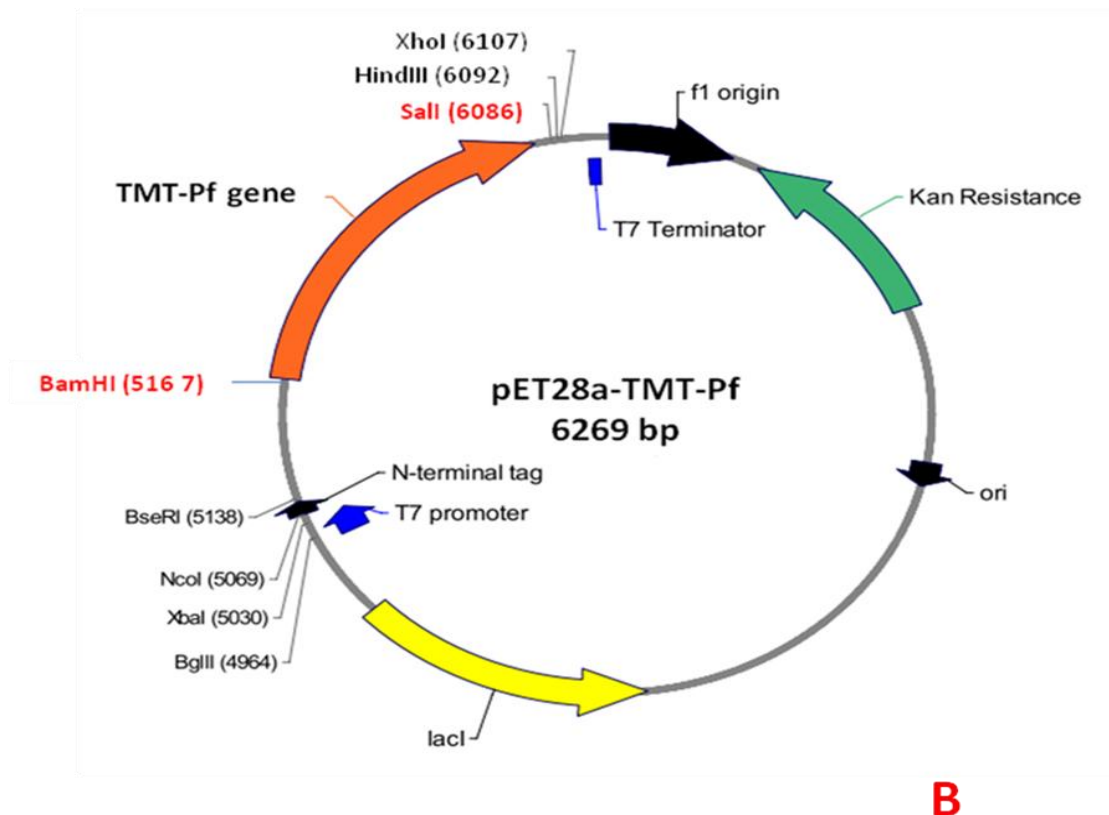


Figure 2. Contd.

at  $-10^{\circ}\text{C}$  in the dark. For determination of  $\gamma$ - and  $\alpha$ -tocopherol in samples, the stock solution was in all cases analysed together with the samples, and analyte concentrations in samples were estimated on the basis of peak areas. All samples were analysed in duplicate. The reaction products were extracted according to Sanchez-Machado et al. (2002). The residue was re-dissolved in 1 ml of the high performance liquid chromatography (HPLC) mobile phase (methanol-acetonitrile, 30:70 v/v), then membrane filtered (pore size  $0.45\ \mu\text{m}$ ; Millipore, USA). Finally, a  $20\ \mu\text{l}$  aliquot was injected into the HPLC column. Before injection, the extracts were maintained at  $-10^{\circ}\text{C}$  in the dark.  $\alpha$ -Tocopherol content were analyzed by 1100 high-performance liquid chromatography apparatus (Agilent Technologies, USA) with a Hypersyl ODS2-C18 column ( $4.6 \times 250\ \text{mm}$ ,  $5\ \mu\text{m}$  particle size), diode array detector (DAD), and a quaternary pump system. HPLC separation was carried out using methanol-acetonitrile (30:70 v/v) as mobile phase. The column was eluted with mobile phase at a flow rate of  $1.0\ \text{ml/min}$ . The column was adjusted to  $30^{\circ}\text{C}$ . The detection was by diode-array detector at a wavelength of  $205\ \text{nm}$ .

## RESULTS

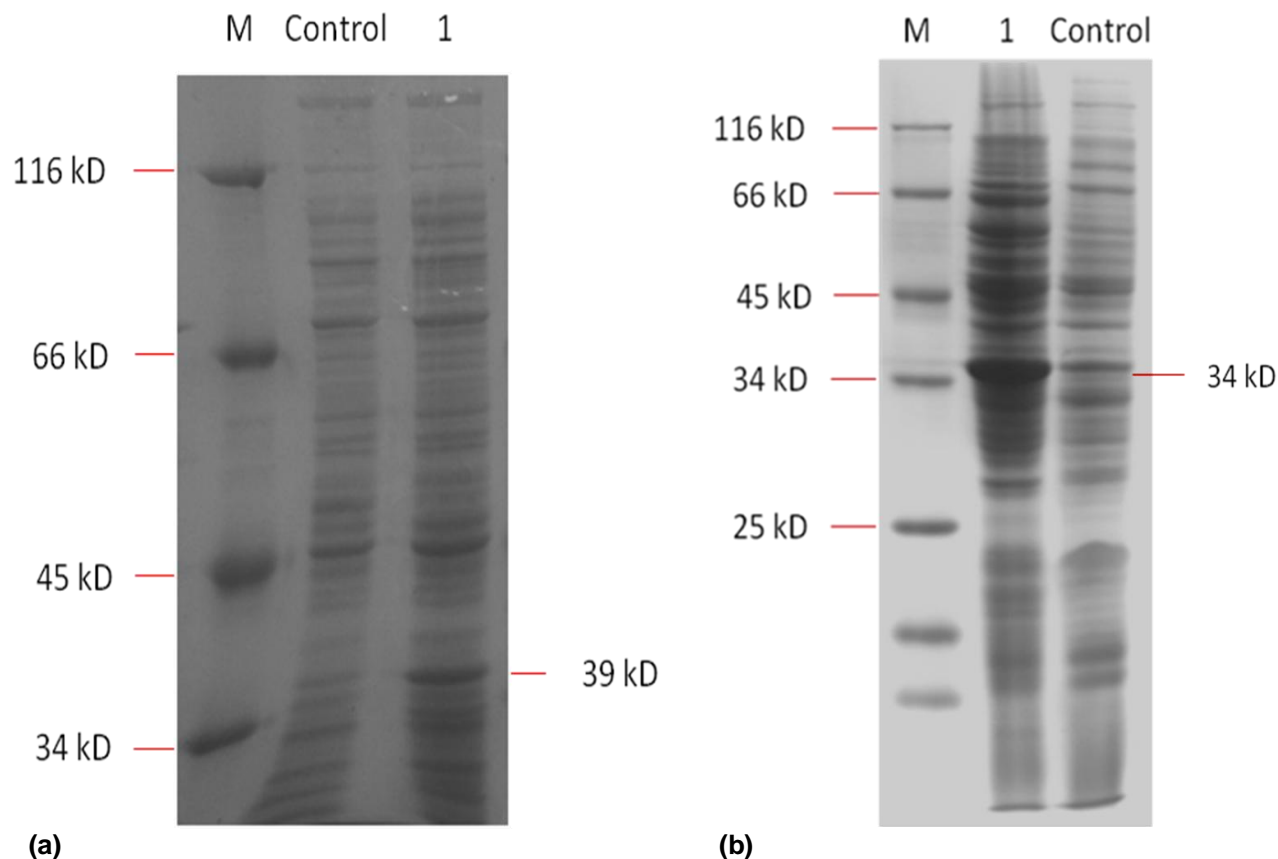
### Characterization of BoTMT

Several pairs of primers were designed according to the conserved mRNA sequences of 5'-end and 3'-end translated regions (GenBank accession number EU637013.1; AF381248.1; AEP68180.1). RT-PCR products, by primers

P1BoTMT/P2BoTMT for Chinese cabbage was sequenced and aligned. The alignment data showed that the sequence was consistent with  $\gamma$ -TMTs. The BoTMT open reading frame of 1044 bp fragment, with primers P3BoTMT/P4BoTMT, encodes a predicted peptide of 347 amino acid residues to produce a truncated protein (39 kD) devoid of a majority of the putative N-terminal signal sequence. The closest match in the databases to the deduced amino acid sequence is a putative  $\gamma$ -TMT, from *B. napus* (GenBank accession number EU637013.1), sharing 98% identity with BoTMT.

### Expression of $\gamma$ -TMTs in *E. coli*

The open reading frame of BoTMT was carried out with primers P3BoTMT/P4BoTMT. The *P. frutescens*  $\gamma$ -TMT, open reading frame of 894 bp fragments, with primers PfTMT/PfTMT (GenBank accession number JN381069.1), encodes a predicted peptide of 297 amino acid residues to produce a protein (34 kD). In order to study the possible function of BoTMT, the expression vector pET28a-TMT-Bo and pET28a-TMT-Pf were constructed and transformed into *E. coli* BL21(DE3). After induction with IPTG, the specified protein band of BoTMT and *P. frutescens*  $\gamma$ -TMT were observation by SDS-PAGE



**Figure 3.** SDS-PAGE of the recombinant BoTMT and *P. frutescens*  $\gamma$ -TMT in *E. coli*. (a) M, molecular marker, the sizes of marker are shown on the left; 1, total protein extracted from *E. coli* BL21 (DE3)/pET-BoTMT; control, total protein extracted from *E. coli* BL21 (DE3)/pET28a. (b) M, molecular marker, the sizes of marker are shown on the left; 1, total protein extracted from *E. coli* BL21 (DE3)/pET-PfTMT; control, total protein extracted from *E. coli* BL21 (DE3)/pET28a.

(Figure 3), which had the same molecular weight of the recombination protein with a 6-His tag sequence of pET28a, while negative control did not produce these bands.

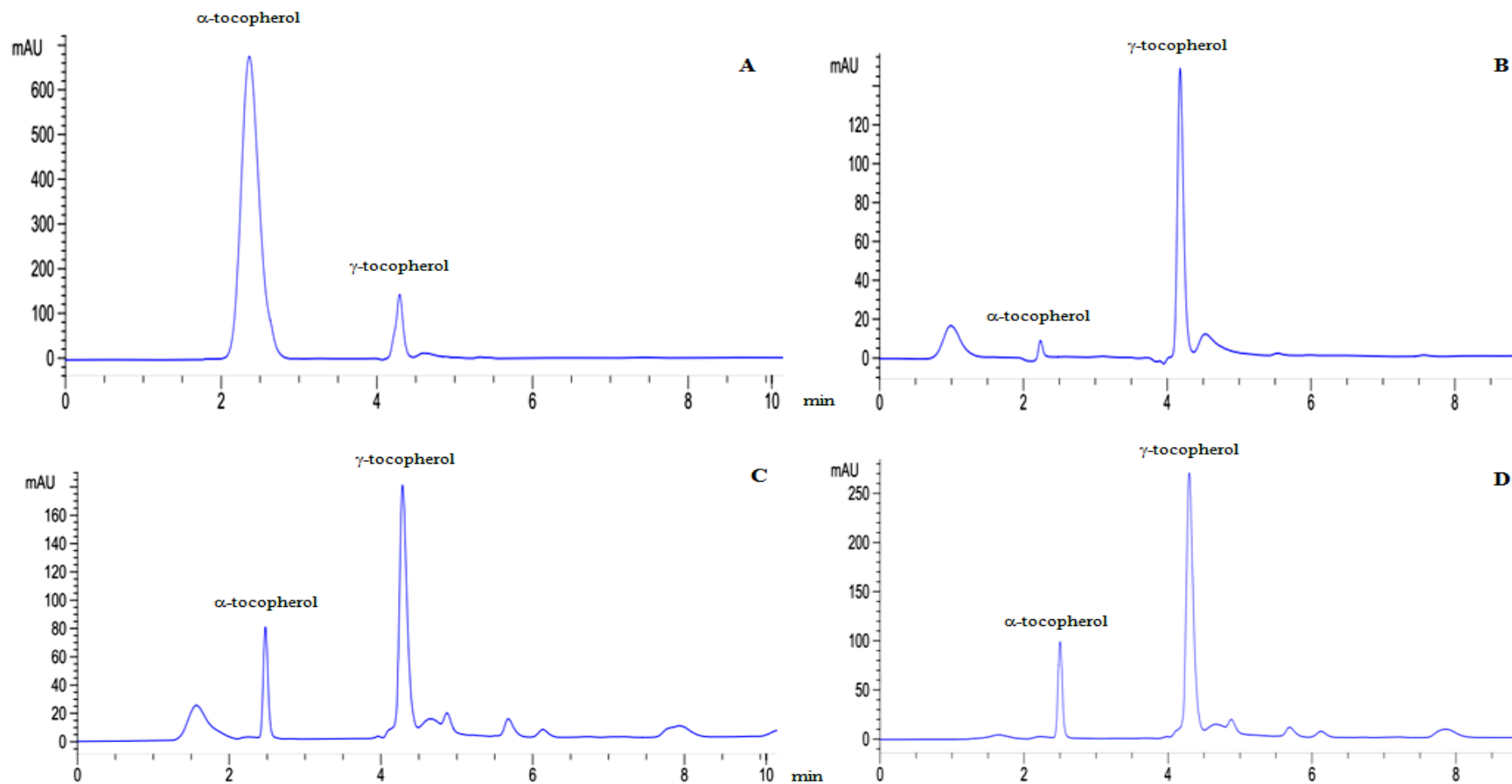
#### The enzyme activity assay of the recombinant $\gamma$ -TMT protein

Quantitative HPLC with ultraviolet detection is currently used for the determination of  $\alpha$ -tocopherol in reaction products. In this study, we used a simple HPLC method for determination of  $\alpha$ -tocopherol. After optimization of the HPLC conditions, peaks of  $\gamma$ - and  $\alpha$ -tocopherol were observed at 4.285 and 2.451 min for UV detection (Figure 4), respectively. Standards of  $\gamma$ - and  $\alpha$ -tocopherol, with different concentrations, were analysed by HPLC for calculating the calibration curve, peak area =  $b \times$  concentration +  $m$ . The protein from *E. coli* BL21/pET28a controls, BL21/pET28a-TMT-Bo and BL21/pET28a-TMT-Pf were added into a reaction system containing  $\gamma$ -tocopherol and SAM. The reaction products were

analyzed by HPLC. The result shows that the  $\alpha$ -tocopherol levels in the reaction products with expression of the BoTMT and *P. frutescens*  $\gamma$ -TMT increased compared to the *E. coli* BL21 controls (Figure 4). In the *E. coli* BL21/pET28a-TMT-Bo and BL21/pET28a-TMT-Pf, the  $\alpha$ -tocopherol contents (and  $\gamma$ -tocopherol conversion yield) in the reaction products were about 23 and 18%, respectively.

#### DISCUSSION

In this paper, a new  $\gamma$ -TMT gene, BoTMT, has been cloned successfully from Chinese cabbage. Expression of BoTMT had been achieved in *E. coli* and the recombinant  $\gamma$ -TMT had the same capability of *P. frutescens*  $\gamma$ -TMT to catalyze the methylation of carbon 5 of  $\gamma$ -tocopherol to produce  $\alpha$ -tocopherol, whereas BL21/pET28a had no activity (Figure 4). The  $\gamma$ -TMT is one of important enzymes in determining the tocopherol composition (Ajjawi and Shintani, 2004). As individual tocopherols have different properties, a detailed



**Figure 4.** HPLC analysis of  $\alpha$ -tocopherol production in recombinant *E. coli* cells. (A) Separation of  $\alpha$ - and  $\gamma$ -tocopherol product standards, (B) *E. coli* BL21(DE3)/pET30a controls, (C) *E. coli* BL21(DE3)/pET-BoTMT transformation, (D) *E. coli* BL21(DE3)/pET-PfTMT transformation.

characterization of further enzymic steps in the tocopherol biosynthetic pathway such as shown here for  $\gamma$ -TMT will be fundamental to support the rational design of engineered crop plants with

modified profiles of tocopherols (Koch et al., 2003), and characterization of  $\gamma$ -TMT can be applied to elevate the levels of this important antioxidant vitamin in the major oilseed crops in the future.

Our future work will focus on improving the present BoTMT expression in soybean by our *Agrobacterium*-mediated transformation and expression vectors (Lv et al., 2012) for the

improved vitamin E content.

### Nucleotide sequence accession numbers

The GenBank accession number for the BoTMT and PftTMT identified in this study are JQ031515 and JN381069.

### ACKNOWLEDGEMENTS

This work was supported by National Basic Research Program of China (973, Program NO.2012CB721103), National High Technology Research and Development Program of China (863, Program NO.2012AA101806), and Priority Program of Development and Application of Biotechnology in Agriculture and Rural Development Towards 2020 of Vietnam.

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