TOCOPHEROL *γ-tmt* GENE ISOLATION, SEQUENCE ANALYSIS FROM *Brassica oleracea* AND *Perila frustescens*

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ABSTRACT

Tocopherols, with antioxidant properties, are synthesized by photosynthetic organisms and play important roles in human and animal nutrition. In the major oilseed crops, γ-tocopherol, the biosynthetic precursor to α-tocopherol, is the predominant form found in the leaves. This suggests that the final step of the α-tocopherol biosynthetic pathway is catalyzed by γ-tocopherol methyltransferase (γ-TMT). The full-length cDNA (BoTMT) and cDNA (PfTMT) of ***γ****-TMT are obtained from Brassica oleracea and Perilla frustescens by RT-PCR (reverse transcriptase-polymerase chain reaction) from the total RNA of leaves. Results of sequence analysis indicate that the cDNA (BoTMT) sequence consisted of an open reading frame of 1041 nucleotides encoding a protein of 347 amino acid residues with a calculated molecular weight of 39 kD polypeptide. cDNA (PfTMT) sequence consisted of an open reading frame of 894 nucleotides encoding a protein of 297 amino acid residues with a calculated molecular weight of 34 kD polypeptide. The result also showed an identity of about 88% between the predicted amino acid* ***γ****-TMT gene sequences derived from Brassica oleracea and Arabidopsis, 72% between Perilla frustescens and Arabidopsis, and 70% between Brassica oleracea and Perilla frustescens. These results demonstrated that the E. coli BL21 expression of the Brassica oleracea and the Perilla frustescens γ-TMT gene resulted in the tocopherol composition from 8.9 to 10.5-fold increase in α-tocopherol content. The increase in the α-tocopherol content indicates a regulatory function of the γ-TMT protein to convert γ-tocopherol to α-tocopherol.*

Keywords: *Brassica oleracea, Perilla frustescens*, γ-TMT, tocopherol, HPLC.

INTRODUCTION

α-tocopherol is considered the most important form of vitamin E for human health, as it has ten-fold higher antioxidant activity than other tocopherols (Traber and Sies 1996). γ-tocopherol has received little attention since the discovery of vitamin E. However, recent studies indicate that γ-tocopherol may also be important to human health and that it possesses unique features that distinguish it from α-tocopherol, such as anti-inflammatory and anticancer activity. However, the bioavailability and bioactivity of γ-tocopherol are lower than those of α-tocopherol (Jiang and Ames 2003; Jiang et al. 2009). Thus, α-tocopherol is expected to be a more potent antioxidant than either β- or γ-tocopherol.

Biosynthesis of tocopherols was demonstrated in plastid envelopes (Sall et al. 1980) from precursors originating from the plastidial isoprenoid pathway and from the shikimate pathway, providing the hydrophobic phytyl moiety and the polar head group homogentisic acid, respectively. Furthermore, plastidial

tocopherol accumulation appears to depend on the up-regulation of genes encoding the enzymes being involved in the formation of these precursors, like 1-deoxy-d-xylulose 5-phosphate synthase (Bouvier et al. 1998), geranylgeranyl reductase (Keller et al. 1998) and 4-hydroxyphenylpyruvate dioxygenase (Norris et al. 1998). Based on earlier investigations and on detailed work on the chemical synthesis of prenylquinones (Mayer et al. 1971) the pathway for plastidial α-tocopherol biosynthesis has been elucidated (Soll et al. 1980; Soll et al. 1985). The proposed pathway includes the phytylation of homogentisic acid to form 2-methyl-6-phytylquinol, the first ring methylation at position 3 to yield 2,3-dimethyl-5-phytylquinol, cyclization to yield γ-tocopherol, and finally a second ring methylation at position 5 to yield α-tocopherol (**Figure 1**).

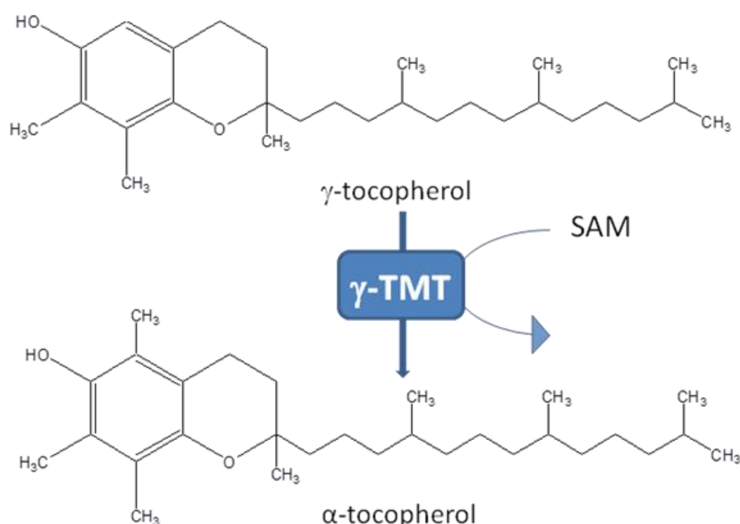

Figure 1. *γ-TMT* enzymatic reaction. *γ-TMT* adds a methyl group to ring carbon 5 of γ-tocopherol.

Studies have been carried out to purify and characterize *γ-TMT* from plants (D'Garkubgye 1985; Koch 2003; Ishiko 1992; Shigeoka 1992; Michalowski 1993) since 1985, but little progress has been made because of its membrane-bound nature, its low amounts in cells and its instability after detergent solubilization (Soll 1985). *Arabidopsis* plastoglobuli were also shown to contain tocopherol cyclase (TC) (Austin et al. 2006; Vidi et al. 2006; Ytterberg et al. 2006), indicating that part of the tocopherol biosynthetic pathway is localized to plastoglobuli. Recently, two *γ-TMT* genes were identified in model organisms *Arabidopsis* and *Synechocystis* through a genomics-based approach, and overexpression of *Arabidopsis* seeds shifted oil composition in favor of α-tocopherol (Shintani 1998), transgenic perilla (Byoung 2008) transgenic soybean (Tavva et al. 2007) and transgenic *Brassica juncea* (Yusuf and Sarin 2007). Overexpression of the *Arabidopsis* enzyme with a seed-specific promoter resulted in a more than 80-fold increase of α-tocopherol at the expense of γ-tocopherol without changing the total content.

These observations suggest that the final enzyme γ-tocopherol methyltransferase (*γ-TMT*) of the α-tocopherol biosynthesis pathway, which catalyzes the methylation of γ-tocopherol to form α-tocopherol, is likely limited in the seeds of the most agricultural important crop. Methylation of γ-tocopherol to form α-tocopherol by chemical catalysis *in vivo* will not only increase the production cost but also bring some other by-products that are harmful to human health. However, the relationship

between the expression pattern of the *γ-TMT* gene and the content of α-tocopherol in plant organs is poorly understood. Understanding the biochemical pathway of tocopherol biosynthesis, therefore, opens the perspective for improving the nutritional quality of crop plants (Grusak 1999). Therefore, regulating the expression of *γ-TMT* through gene engineering will help to understand the γ-tocopherol biosynthesis pathway; and has a potential contribution to human health.

In this study, the full-length cDNA of *γ-TMT* was obtained from *B. oleracea* (named *BoTMT*) and *Perilla fruscestens* (named *PfTMT*), and these deduced amino acid sequences were compared with other organisms. The research team attempted a detailed characterization of *γ-TMT* activities with respect to substrate specificities expressed in *E. coli.*

MATERIALS AND METHODS

Plant materials

Leaves of ball cabbage (*Brassica oleracea*) and *Perilla frutescens* were used to isolate total RNA.

Strains, plasmid, and major reagent

E. coli DH5α, BL21 were from Fermentas (China). RNAiso for Polysaccharide-rich Plant Tissue, PrimeScript™ II 1st strand cDNA Synthesis Kit were from TaKaRa (Dalian, China). The sequencing vector pMD19-T was from Jieli Biotech (Shanghai, China). DNA Gel Extraction Mini Kit was purchased from Axygen Company. Restriction endonuclease, T4 DNA ligase, and Taq DNA polymerase were from TaKaRa (Dalian, China). PCR primers were synthesized by Sangon (Shanghai, China). γ-, α-tocopherols, S-Adenosylmethionine (SAM) was purchased from Sigma (Deisenhofen, Germany). Chromatographic materials and columns were obtained from Agilent Technologies, USA. All other chemical reagents were of analytical purity.

Primer design

The following sequences are designed by Primer Premier 5.0 software.

Total RNA extraction

The used solutions and reagents were as follows: extraction buffer: RNAiso™ for Polysaccharide-rich Plant tissue solution (Takara, Japan), chloroform/isoamyl alcohol (24:1, v/v), high salt solutions (0.8M sodium citrate + 1.2M sodium chloride), 5M sodium chloride, isopropanol, ethanol (75%, v/v), diethylpyrocarbonate (DEPC) treated water. All solutions must be kept RNAse-free, leaves were harvested from *Brassica oleracea* and *Perilla frutescens* plant 10-15 days after seeding, frozen in liquid nitrogen, and stored at -70ºC. The RNA extraction protocol was used by RNAiso™ for Polysaccharide-rich Plant tissue solution (Takara, Japan) according to the manufacturer's instructions. Five microliters of RNA solution were diluted to be qualitatively assessed with a Nanodrop 2000 spectrophotometer and tested on a 1.1% formaldehyde denaturant agarose gel.

Reverse Transcriptase Reactions PCR

For *γ-TMT* gene amplification, total RNA was isolated from leaves of *Brassica oleracea,* and *Perilla frustescens* were used for 1st strand cDNA synthesis reaction by using PrimeScript™ II 1st strand cDNA Synthesis Kit (TaKaRa) according to the manufacturer's instructions. RNA samples were vacuum dried and used for RT (reverse transcriptase) reactions. For *Brassica oleracea*, a PCR mixture containing Premix Taq Version 2.0 (TaKaRa), template DNA, primer P1BoTMT, and primer P2BoTMT (**Table 1**) was used. For *Perilla frutescens*, a PCR mixture containing Premix Taq Version 2.0 (TaKaRa), template DNA, primer P1PfTMT, and primer P2PfTMT (**Table 1**) was used. The experimental conditions of RT-PCR were 95ºC for 5 min, then 30 cycles of 95ºC 30s, 55ºC 30s, and 72ºC 1.5 min, followed by a final extension at 72ºC for another 10 min.

Table 1. Primers used in this study.

Primers	**Sequences (5'-3')**
P1BoTMT	GAACTTAGAGAGGCTTCTGGCAA
P2BoTMT	CGGGATCCACCATGAAAGCGACTCTCG
P3BoTMT[a]	GGATCCG**ATG**GCGGAGATGGAGACGGA
P4BoTMT[b]	GTCGAC**CTATTA**GAGAGGCTTCTGGCAAGTGAT
P1PfTMT	CCATGGCGGAGATGGAGACGGAGATGGAG
P2PfTMT	CTCGAGAGATGCAGGTTTTCGGCATGTA
P3PfTMT[a]	GGATCCG**ATG**GCGGAGATGGAGACGGA
P4PfTMT[b]	GTCGAC**TTA**AGATGCAGGTTTTCGGCATGTAATG

[a]The *Bam*HI restriction site is underlined, and the start codon site is bold.
[b]The *Sal*I restriction site is underlined, and the stop codon site is bold.

Transformation of the plasmid into DH5a, BL21 strain of *Escherichia coli*

Amplification products were fractionated on 1% agarose gel from which the selected band was purified. The amplified DNA was inserted into the pMD19-T vector (TaKaRa) and transformed into *E. coli* DH5α. One hundred microlitres of bacteria stock solution were mixed with 100ng plasmid, chilled on ice for 30 min, and put in a 42ºC water bath for 2 min. The mixture was put immediately on ice again for about 2 min, incubated in LB broth in a shaking incubator (160 rpm) for 60 min at 37ºC and streaked onto different LB agar plates, each containing ampicillin (for DH5a) or kanamycin (for BL21) the concentration of 100 µg/mL. The plates were incubated overnight at 37ºC and analyzed. The positive plaques were identified by PCR and then sequenced by Jieli Biotech (Shanghai, China). The negative control would be an untransformed *E. coli* strain. The bacteria plate was sub-cultured once every week to make sure that the bacteria were fresh before every plasmid extraction process.

Culture of *E. coli* in LB broth

A colony of the transformed *E. coli* was picked and mixed into antibiotic-containing LB broth. (in g/L: tryptone 10.0, yeast extract 5.0, NaCl 5.0; pH 7.2-7.5) at 37°C on an orbital shaker (180 rpm) for 12-16 h. In the case of the recombinant strains, LB medium was supplemented with ampicillin (100 mg/mL) for the DH5a strain or kanamycin (100 mg/mL) for the BL21 strain. Plates containing LB medium supplemented with agar (16 g/L) were incubated at 37°C for 12-16 h. The broth was then incubated in a shaking incubator (150 rpm) at 37°C overnight prior to being used for plasmid DNA extraction.

Vector construction

The full coding region of *BoTMT* and *PfTMT* genes was amplified by Polymerase Chain Reaction (PCR) using pBoTMT and pPfTMT plasmid as templates. For the *BoTMT* gene, the upstream primer P3BoTMT (**Table 1**) was underlined as *a Bam*HI site and a translational start codon. The downstream primer was P4BoTMT (**Table 1**) underlined are the *Sal*I site and two stop codons. For *PfTMT*, the upstream primer P3PfTMT (**Table 1**) was underlined as *a Bam*HI site and a translational start codon. The downstream primer was P4PfTMT (**Table 1**) underlining the *Sal*I site and the stop codon. The PCR products were cloned into the pMD19-T vector to generate pBoTMT and pPfTMT. Both pBoTMT and pPfTMT were digested with *Bam*HI and *Sal*I. The fragments of the product were purified and cloned into the vector pET28a (TaKaRa) to obtain the express vector pET28a-TMT-B and pET28a-TMT-Z (**Figure 2 and Figure 3**). The plasmids were then transformed into *E. coli* BL21 by heat shock transformation.

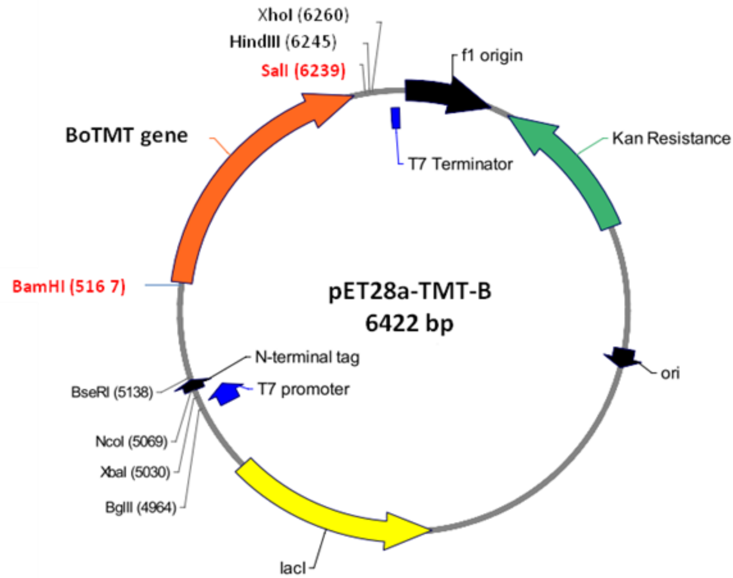

Figure 2. Schematic diagram of the binary vectors pET28a-TMT-B (6,422 bp).

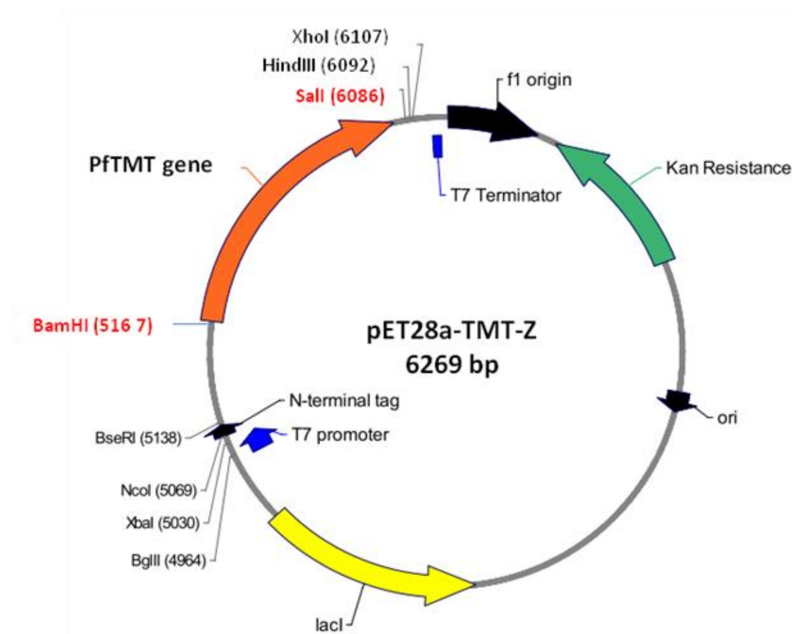

Figure 3. Schematic diagram of the binary vectors pET28a-TMT-Z (6,269 bp).

Expression *BoTMT* and *PfTMT* gene in *E. coli* BL21

The transformants harboring plasmid pET28a-TMT-Bo and pET28a-TMT-Pf were cultured at 37ºC in LB medium until OD_{600nm} reached 0.5-0.7. IPTG was added to the final concentration of 0.5 mmol/L and the cultivation was continued for another 4-5 hours at 37ºC. The cell was harvested by centrifugation. The protein concentration was measured by Bio-Rad Protein Assay. Total bacterial protein was run at 12% SDS-PAGE.

The enzyme activity assay of the recombinant *γ-TMT*

Protein expression was induced as described above, and the growth temperature was cultured at 37°C in an LB medium. Extracts from *E. coli* BL21-induced cells carrying on pET28α-TMT-B, pET28α-TMT-Z, and pET28a respectively, were harvested by centrifugation. The protein concentration of the supernatant was determined by Nanodrop 2000, and 100pl of supernatant was used for *γ-TMT* activity analysis. The assay for the *γ-TMT* enzyme is based on the methylation of exogenous γ- into α-tocopherol in the presence of SAM. A *γ-TMT* activity assay was performed as described by Shitani et al. (1998) showed that the final concentrations of γ-tocopherol and SAM were 0.02 mmol/L and 1 mmol/L, respectively. The enzyme activity was measured by assessing the residual enzyme activity after incubating in a shaking incubator (150 rpm) at 30°C for 10 h.

Chemical analysis

The α-tocopherol stock was prepared by dissolving 30 mg of α-tocopherol in 100 mL of methanol-acetonitrile (30:70 v/v), giving a final concentration of 300 mg/mL. The stock was used to obtain working solutions of 0.75, 1.5, 3.0, and 6.0 µg/mL, which were stored at -10°C in the dark. For the determination of α-tocopherol in samples, the stock solution was in all cases analyzed together with the samples, and analyte concentrations in samples were estimated on the basis of peak areas. All samples were analyzed in duplicate.

The reaction products were extracted according to D.I. Sanchez-Machado et al. (2002). The residue was redissolved in 1 ml of the HPLC mobile phase (methanol–acetonitrile, 30:70 v/v), then the membrane filtered (pore size 0.50 mm; Millipore, Bedford, MA, USA). Finally, a 20 µL aliquot was injected into the HPLC column. Before injection, the extracts were maintained at -10°C in the dark. α-tocopherol content was analyzed by 1100 high-performance liquid chromatography apparatus (Agilent Technologies, USA) with a Hypersyl ODS2-C18 column (4.6×250 mm, 5µm particle size), DAD detector, and a quaternary pump system. HPLC separation was carried out using methanol-acetonitrile (30:70 v/v) as the mobile phase. The column was eluted with the mobile phase at a flow rate of 1.0 mL/min. The column was adjusted to 30°C. The detection was by a diode-array detector at a wavelength of 205 nm.

RESULTS AND DISCUSSION

Characterization of *γ-TMT Brassica oleracea* (*BoTMT*) and *γ-TMT Perilla frustescens* (*PfTMT*)

Several pairs of primers were designed according to the cDNA sequences of 5'-end and 3'-end translated regions. RT-PCR products only by primers P1BoTMT/P2BoTMT (**Table 1**) for *Brassica oleracea* and P1PfTMT/P2PfTMT (**Table 1**) for *Perilla frustescens* were obtained from leaves. Then PCR products of the same length were amplified from the *Brassica oleracea* and *Perilla frustescens* genomes, respectively. Analysis of the sequences showed that all of *Brassica oleracea* and *Perilla frustescens* were 1044 bp, and 894 bp (**Figure 4 and Figure 5**), and the sequences were consistent, indicative of having no intron in the *BoTMT* and *PfTMT* gene. These differences may be the results either of amplification artifacts resulting from the inherent inaccuracy of DNA polymerase, or of some sequencing error. The *BoTMT* open reading frame of 1044 bp fragment encodes a predicted peptide of 347 amino acid residues with a molecular weight of 39 kD. The *PfTMT* open reading frame of 894 bp fragments encodes a predicted peptide of 297 amino acid residues with a molecular weight of 34 kD. Alignment of the deduced amino acid sequences of *γ-TMT* gene in *Brassica oleracea* and *Perilla frustescens* in **Figure 7** shows an identity of about 88% between the predicted amino acid sequences derived from *Brassica oleracea γ-TMT* gene and *Arabidopsis*, 72% between *Perilla frustescens γ-TMT* gene and *Arabidopsis,* 70% between *Brassica oleracea γ-TMT* and *Perilla frustescens γ-TMT*. The

Glycine max *γ-TMT* protein shares a high degree of amino acid sequence similarity with *Perilla frustescens γ-TMT* (74%) and *Brassica oleracea γ-TMT* (64%).

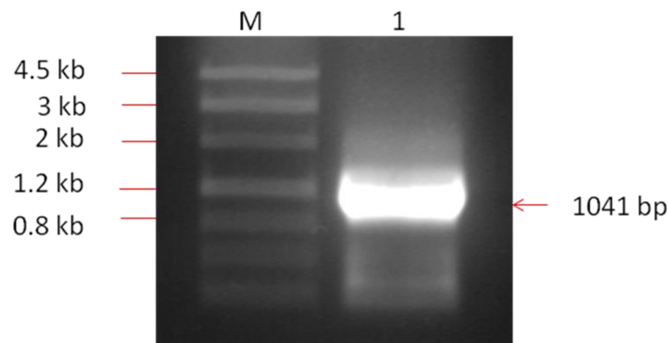

Figure 4. PCR amplification of *BoTMT* gene from *Brassica oleracea* genomic DNA. M: marker IV; 1: PCR product (1041 bp).

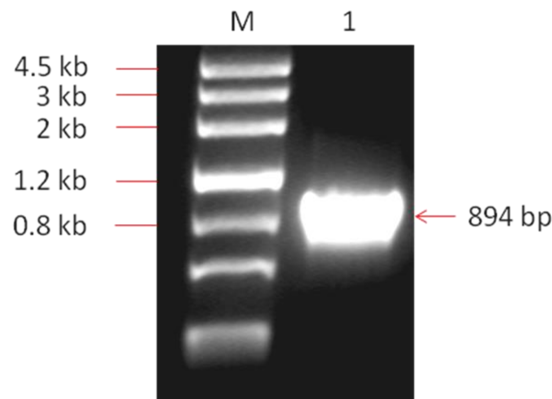

Figure 5. PCR amplification of *PfTMT* gene from *Perilla frustescens* genomic DNA. M: marker IV; 1: PCR product (894 bp).

Expression of *γ-TMT* in *E. coli*

The deduced amino acid sequence of *γ-TMT* from *Brassica oleracea* and *Perilla frustescens* indicates the presence of a putative plastidial transit peptide in the preprotein. The plastidial transit peptide induces the preprotein transporting into the plastids. The plastidial processing of the preprotein in the removal of the targeting peptide is necessary to yield a mature enzyme. This N-terminal signal sequence could affect the conformation of *γ-TMT* protein when expressed in *E. coli* and render the protein inactive. Therefore, a 1044 bp sequence of *γ-TMT* of *Brassica oleracea* was also modified by PCR using a pair of primers P3BoTMT and P4BoTMT to produce a truncated protein (39 kD) devoid of a majority of the putative N-terminal plastidial signal sequence. Besides that, an 894 bp sequence of *γ-TMT* of *Perilla frustescens* was also modified by PCR using a pair of primers P3PfTMT and P4PfTMT (**Table 1**) to produce a truncated protein (34 kD) devoid of a majority of the putative N-terminal plastidial signal sequence.

In order to study the possible function of *γ-TMT* from *Brassica oleracea* and *Perilla frustescens*, the prokaryotic expression vector pET28a-TMT-B and pET28a-TMT-Z were constructed

and transformed into *E. coli* BL21 (DE3). After induction at 37ºC for 4h with IPTG, the specified protein band of vector pET28a-TMT-B and pET28a-TMT-Z were observed in 12% SDS-PAGE, which had the same molecular weight of the recombination protein with a 6xHis tag sequence of pET28a (**Figure 6A**, lane 1 and **Figure 6B**, lane 1), while negative control did not procedure this band (**Figure 6A**, lane control, and **Figure 6B**, lane control).

The enzyme activity assay of the recombinant *γ-TMT* protein

Quantitative HPLC with ultraviolet detection is currently used for the determination of α-tocopherol in reaction products. In this study, we used a simple HPLC method for the determination of α-tocopherol. After optimization of the HPLC conditions, peaks of γ- and α-tocopherol were observed at 4.285 min and 2.451 min for UV detection. In HPLC analysis of γ- and α-tocopherol standards, the peaks likewise eluted at these times, and showed very similar excitation and absorbance spectra (**Figure 5**), indicating that the peaks corresponded to γ- and α-tocopherol.

Four different solutions of known concentrations of analyte included between 0.75 and 6.0 μg/mL. Solutions for the calibration curve were prepared by the same procedure as the samples and with concentrations of α-tocopherol. The curve equation $y = bx + m$ calculated with linear regression method to determine the reaction products concentration was utilized. It is possible to evaluate model effectiveness by R^2 value and by spreads between known concentration and concentration calculated on the calibration curve. Results showed that the equation of the curve with $y = 216x - 9.764$ and the R^2 value (0.998) shows the good linearity of the analytical method under examination.

The recombinant *γ-TMT* protein from *E. coli* BL21/pET28a controls, BL21/pET-BoTMT, and BL21/pET-PfTMT were added into a reaction system containing γ-tocopherol and SAM. The reaction products were analyzed by HPLC. The result showed that the reaction products contents in enzyme from the *E. coli* BL21/pET28a control overexpression of the *BoTMT* and *PfTMT* gene increased the α-tocopherol levels compared to the *E. coli* BL21 controls (**Figure 8**). In the *E. coli* BL21/pET28a controls, the α-tocopherol contents in the reaction products were 0.19 μg/ml, respectively. In the *E. coli* BL21/pET-BoTMT and BL21/pET-PfTMT, the α-tocopherol contents in the reaction products were 1.7 μg/ml and 2.0μg/mL, respectively. On average, an 8.9-fold increase in the α-tocopherol content from *E. coli* BL21/pET-BoTMT transformation and a 10.5-fold increase in the α-tocopherol content from *E. coli* BL21/pET-PfTMT transformation compared to *E. coli* BL21/pET28a controls, respectively.

The results were carried out using methanol-acetonitrile (30:70 v/v) as the mobile phase because the assays performed to optimize the chromatographic method showed that the 30:70 v/v mix methanol:acetonitrile achieved the best resolution of α-tocopherol (retention time of 2.451 min), managing to separate it from other isomers such as γ-tocopherol. Vitamin E is easily oxidable, oxidation losses can be induced by heat, light, alkaline pH, and the presence of free radicals or other components in samples that can oxidize vitamin E during the extraction process and in the extract until its final analysis. That is the reason why the samples were dried under nitrogen to limited oxidable and before injection to a column, the extracts were maintained at -10ºC in the dark.

These results demonstrate that both enzyme preparations from *E. coli* BL21/pET-BoTMT and BL21/pET-PfTMT showed the conversion of γ- to α-tocopherol, respectively, whereas BL21/pET28a was not accepted as substrate (**Figure 6**). The *γ-TMT* protein expressed in *E. coli* has relatively high enzyme activity to catalyze the methylation step leading of γ-tocopherol to the formation of α-tocopherol, respectively, is exerted by one enzyme. This

observation points to the specific methylation by this enzyme at the C(5)-position (i.e. in ortho-position to the prenyl residue) of the tocopherol aromatic head group, recently described by Shintani and DellaPenna (1998). Methylphytylbenzoquinone methyl transferase, tocopherol cyclase, and *γ-TMT* are the enzymes important in determining the tocopherol composition (Ajjawi and Shintani 2004). As individual tocopherols have different properties, a detailed characterization of further enzymic steps in the tocopherol biosynthetic pathway such as shown here for *γ-TMT* will be fundamental to support the rational design of engineered crop plants with modified profiles of tocopherols. Interplay between already known proteins and yet unknown factors will be elucidated by protein interaction studies using approaches such as the yeast two-hybrid system or pulldown assays. Analysis of transgenic lines and mutants with modified activities of individual components such as *γ-TMT* will enable the study of the regulatory processes of the tocopherol biosynthetic pathway in planta and can be applied to elevate the levels of this important antioxidant/vitamin in the major oilseed crops in the future.

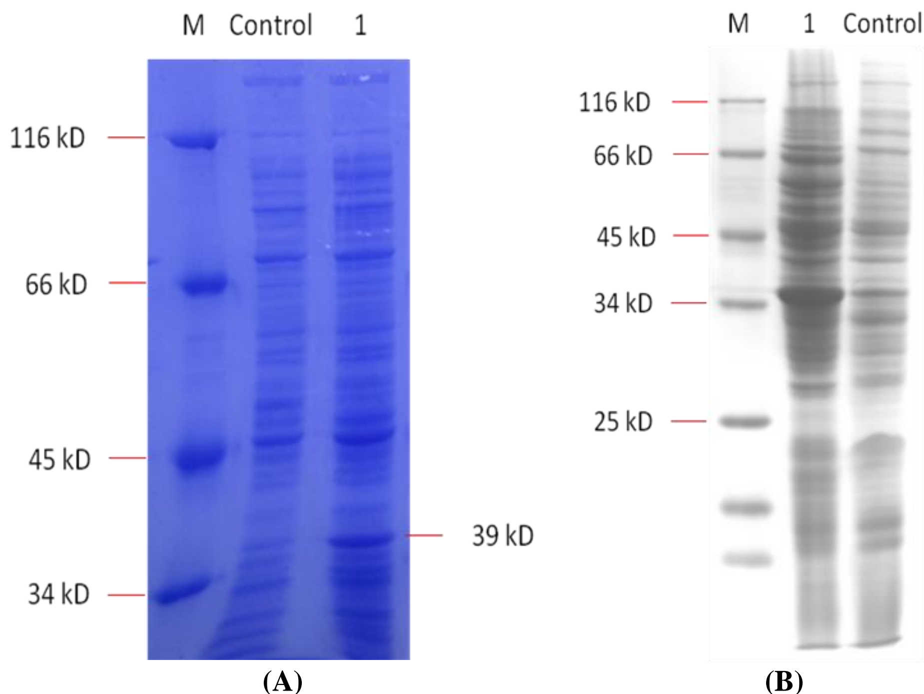

Figure 6. Expression of the recombinant *BoTMT* and *PfTMT* in *E. coli* induced by IPTG (12% SDS-PAGE). **(A)** M, molecular marker, the sizes of markers are shown on the left; 1, total protein extracted from *E. coli* BL21 (DE3)/pET-BoTMT; Control, total protein extracted from *E. coli* BL21 (DE3)/pET28a. **(B)** M, molecular marker, the sizes of markers 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.

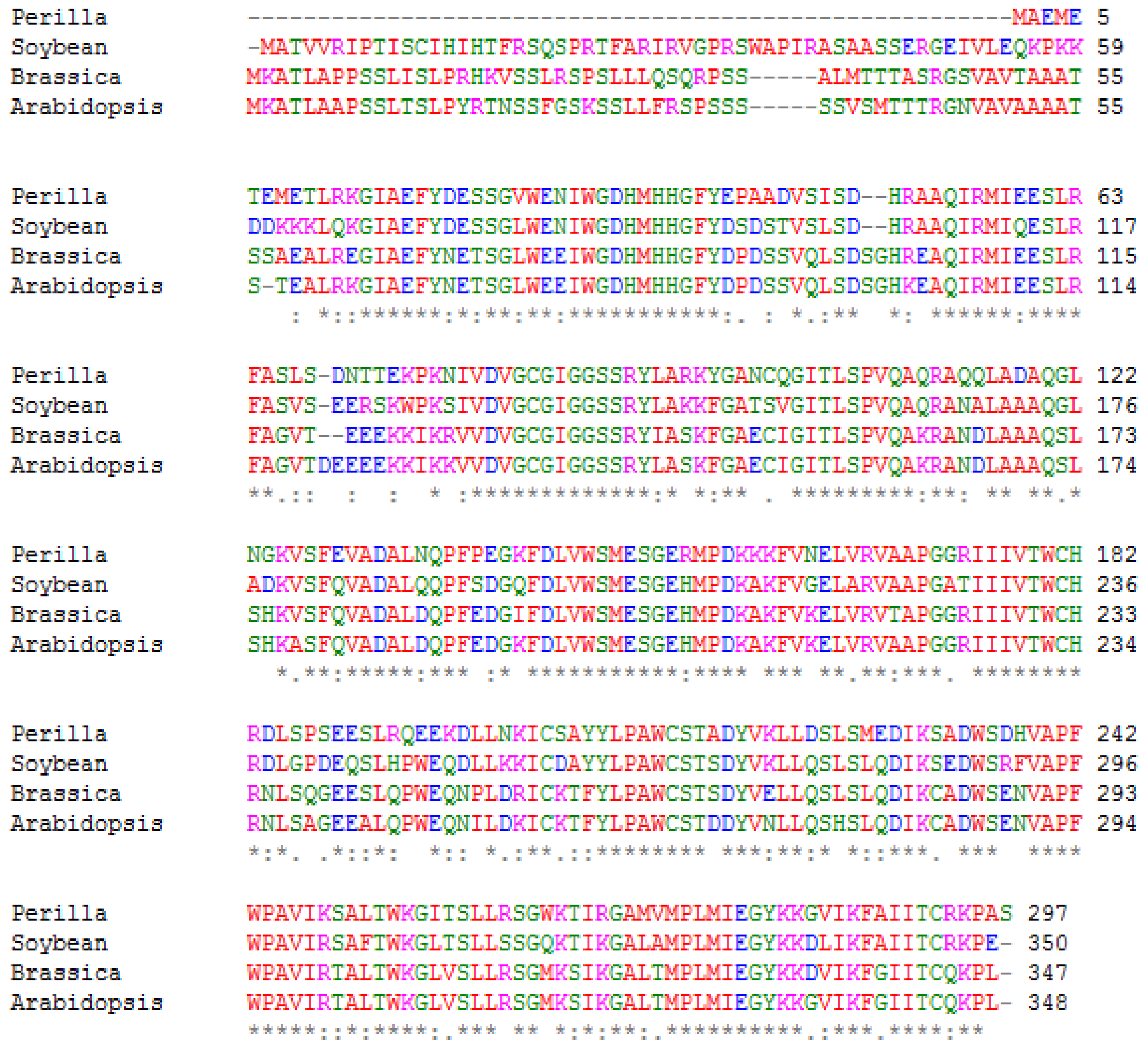

Figure 7. Alignment of *γ-TMT* protein sequences from *Perilla frutescence, Brassica oleracea,* and two other organisms using ClustalW2 software. The deduced amino acid sequences compared are from: *Arabidopsis thaliana γ-TMT* gene (AF104220), *Glycine max γ-TMT* (AY960126), *γ-TMT Brassica oleracea* (this paper), and *γ-TMT Perilla frutescens* (this paper).

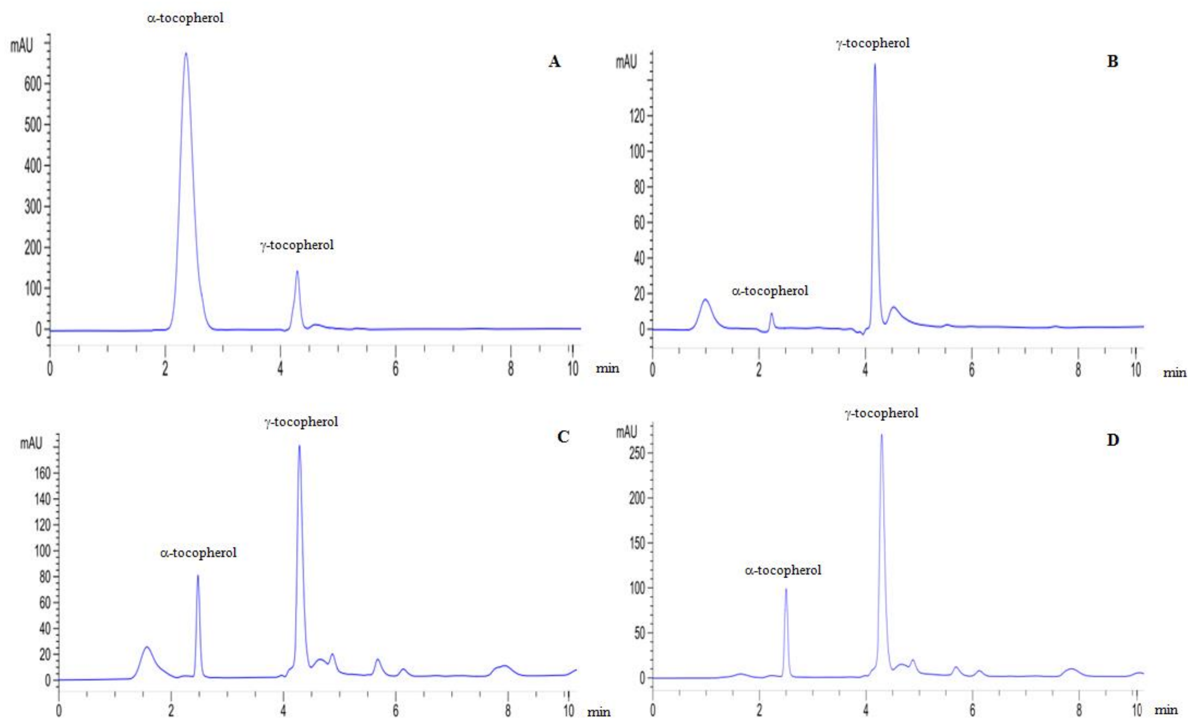

Figure 8. (A) Separation of α- and γ-tocopherol product standards; **(B)** HPLC analysis of α-tocopherol production in *E. coli* BL21(DE3)/pET30a controls. **(C)** HPLC analysis of α-tocopherol production for *E. coli* BL21(DE3)/pET-BoTMT transformation. **(D)** HPLC analysis of α-tocopherol production for *E. coli* BL21(DE3)/pET-PfTMT transformation.

CONCLUSIONS

In this paper, *B. oleracea* and *P. frutescens γ-TMT* gene, has been cloned successfully. Overexpression of *BoTMT* and *PfTMT* has been achieved in *E. coli* and the recombinant *γ-TMT* has the capability to catalyze the methylation of carbon 5 of the tocopherol chromanolring, thus producing α-tocopherol. In the present study, by overexpressing the *B. oleracea* and the *P. frutescens γ-TMT* genes as subtracted, this study was able to improve the tocopherol composition from 8.9-fold to 10.5-fold increase in α-tocopherol content. The recombinant *γ-TMT* will be useful for large-scale catalysis *in vitro*.

COMPETING INTERESTS

The authors declare they have no conflict of interest, financial or otherwise.

AUTHOR'S INFORMATION AND CONTRIBUTIONS

Tran Vu Hai is the person who directly did this research with the guidance of the Professors.

ACKNOWLEDGEMENTS

This work was supported by the Priority Program of Development and Application of Biotechnology in Agriculture and Rural Development Towards 2020, Vietnam.

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PHÂN LẬP VÀ ĐẶC TÍNH CỦA GEN MÃ HÓA *γ-TOCOPHEROL METHYLTRANSFERASE* TỪ CÂY BẮP TÍM (*BRASSICA OLERACEA*) VÀ TÍA TÔ (*PERILA FRUSTESCENS*)

Tocopherols, với đặc tính chống oxy hóa, đóng vai trò quan trọng trong thành phần dinh dưỡng của người và động vật. γ-tocopherol, tiền chất sinh tổng hợp α-tocopherol, được tìm thấy trong lá ở một số loài hạt cây có dầu. Nhiều nghiên cứu cho thấy, giai đoạn cuối cùng của quá trình tổng hợp α-tocopherol được xúc tác bởi γ-tocopherol methyltransferase (γ-TMT). Qua kỹ thuật RT-PCR từ ARN của lá Bắp cải tím và Tía tô, chúng tôi đã phân lập cDNA của gen γ-TMT và đặt tên γ-BoTMT (Genbank: JQ031515) và γ-PfTMT (Genbank: JN381069.1). Trình tự phân tích cho thấy, khung đọc mở của gen γ-BoTMT và γ-PfTMT có chiều dài lần lượt 1041 bp và 894 bp mã hóa đoạn polypeptid có trọng lượng 39 kD và 34 kD. Ngoài ra, kết quả còn cho thấy sự biểu hiện của γ-PfTMT và γ-BoTMT qua vi khuẩn E. coli BL21(DE3) làm tăng hàm lượng α-tocopherol (hiệu suất chuyển đổi γ-tocopherol) từ 18% đến 23% trong các sản phẩm phản ứng tạo thành. Sự gia tăng hàm lượng α-tocopherol chỉ ra chức năng của protein γ-TMT trong việc chuyển đổi γ-tocopherol thành α-tocopherol.

Từ khóa: Tía tô*; Bắp cải tím; Brassica oleracea; Perilla frustescens*; γ-TMT; tocopherol; HPLC.