Cloning and analysis of a γ-tocopherol methyltransferase gene from *Brassica oleracea* and the function of its recombinant protein*

OUYANG Qing[1], FAN Chuntao[2], SUN Hui[1], ZHANG Yuman[1], BAI Shuangyi[3], CAI Wenqi[1]**

(1. Institute of Microbiology, Chinese Academy of Sciences, Beijing 100080, China; 2. College of Life Sciences, Beijing Normal University, Beijing 100875, China; 3. Department of Landscape Architecture, China Agricultural University, Beijing 100094, China)

Received January 24, 2003

Abstract The full-length cDNA (*BoTMT*) of γ-TMT is obtained from *Brassica oleracea* by 3′- and 5′-RACE methods. The 1265 bp cDNA contains an open reading frame of 1044 bp, which encodes a protein of 347 amino acids with a predicted chloroplast transit peptide and two S-adenosylmethionine (SAM)-binding domains. Sequence analysis shows that the deduced protein shares 41.8% ~ 86.5% similarity to known γ-TMTs. Semi-quantitative RT-PCR reveals that *BoTMT* is expressed preferentially in flowers and leaves of *B. oleracea*. The recombinant γ-TMT protein is obtained by cloning its encoding region into the prokaryotic expression vector pET30a. The protein expressed in *E. coli* accounts for 22% of total bacterial protein. The enzyme activity assay indicates that the recombinant protein has relatively high activity to convert γ-tocopherol to α-tocopherol *in vitro*.

Keywords: ***Brassica oleracea*, gamma-tocopherol methyltransferase, gene and expression, enzyme activity assay, tocopherol.**

Tocopherols, commonly known as vitamin E, are a class of lipid-soluble antioxidants synthesized only by plants and other photosynthetic organisms[1]. In addition to their role as antioxidants, tocopherols are essential micronutrients needed for human health. Daily intake of tocopherols (10~13.4 IU) is required for the normal metabolism of muscle, central nervous system, vascular system and reproductive system[2]. Over the past 20 years, epidemiological evidence has indicated that tocopherol supplementation at therapeutic doses (100~1000 IU) contributes significantly to reducing the risk for cardiovascular disease and cancer, improves immune function, and prevents or slows down a number of degenerative disease processes in humans[3,4].

Natural vitamin E is much better than synthetic ones in both biological activity and security. The activity of natural vitamin E is 1.3 to 1.4 fold of those synthesized chemically[5]. Of the four naturally occurring tocopherols, α-tocopherol has the highest vitamin E activity (100, 50, 10 and 3 percent relative activity for α-, β-, γ-, δ-tocopherol, respectively) and is preferentially absorbed and distributed throughout the body[6]. Plant oils are the main dietary source of tocopherols[5]. However, α-tocopherol is present only as a minor component (7% ~ 10%) while its biosynthetic precursor, γ-tocopherol, is present at high level (67% ~ 70%)[5,7]. These observations suggest that the final enzyme γ-tocopherol methyltransferase (γ-TMT) of the α-tocopherol biosynthetic pathway, which catalyzes the methylation of γ-tocopherol to form α-tocopherol, is likely limited in the seeds of most agriculturally important oil crops. Methylation of γ-tocopherol to form α-tocopherol by chemical catalysis *in vitro* will not only increase the production cost, but also bring some other byproducts which are harmful to human health. Therefore, regulating the expression of γ-TMT through gene engineering will help to understand the α-tocopherol biosynthesis pathway, and has potential contribution on human health.

Studies have been carried out to purify and characterize γ-TMT from plants[9~13] 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[8]. Recently, two γ-TMT genes were identified in model organisms *Arabidopsis* and *Synechocystis* through genomics-based approach, and overexpression of γ-TMT in *Arabidopsis* seeds shifted oil compositions in favor of α-tocopherol[14]. By searching the GenBank database, three other amino acids sequences (NP485843, AAM94332, AAL36933) which encode putative γ-TMTs were found. However, the studies

* Supported by the National Natural Science Foundation of China (Grant No. 30270856)

** To whom correspondence should be addressed. E-mail: caiwq@sun.im.ac.cn

on γ-TMT genes from other plants and their applications to non-model plants have not been reported. The relationship between the expression pattern of γ-TMT gene and the content of α-tocopherol in plant organs is poorly understood.

In this study, the full-length cDNA of γ-TMT was obtained from *B. oleracea* (named *BoTMT*) and a semi-quantitative RT-PCR was conducted to analyze the expression of *BoTMT* in various organs of *B. oleracea*. The enzyme was expressed in *E. coli* and functionally characterized.

1 Materials and methods

1.1 Materials

The seeds of ball cabbage (*B. oleracea* L. var. *capitata* L.) ZHONG GAN No. 11 were kindly provided by Prof. Debin Wang (Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences). The shoots of 9-day-old seedlings were used to isolate total RNA. The roots, stems, leaves and flowers used in semi-quantitative RT-PCR were collected from the mature field grown plants. Standard α-, γ-tocopherol, S-adenosyl-L-methionine, butylated hydroxytolulene (BHT), α, α′-dipyridyl were purchased from Sigma Company.

1.2 Cloning of *BoTMT* cDNA

The 3′ RACE primers Psa3 (5′-GAAAGTAGTGGATGTTGGGTG-3′) and Psa4 (5′-CAGGAGGTAGGATAATAATAGTGA-3′) were designed according to the γ-TMT gene sequences of *Arabidopsis* and *Synechocystis*[14]. The 5′ RACE primers GSP1 (5′-TTCCACGTTAATGCGGTTC-3′) and GSP3 (5′-GCAGATTCTGTCCAAGAGGTTC-3′) were synthesized based on the 3′ end sequence of *B. oleracea* γ-TMT cDNA. Oligo (dG) primer AAP (5′-GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG-3′) was used as anchor primer. The reverse transcription product of total RNA from *B. oleracea* was used as template in RACE. Sequence splicing was done by DNASTAR software. The primers RT6 (5′-CGGGATCCACCATGAAAGCGACTCTCG-3′) and RT3 (5′-TGAACTTAGAGAGGCTTCTGGCAA-3′) were designed for cloning the entire coding region. Protein sequences were aligned with GenBank Blastp available at web site http://www.ncbi.nlm.nih.gov/.

1.3 Semi-quantitative RT-PCR

The total RNAs of root, stem, leaf, flower and seed of *B. oleracea* were extracted using Trizol reagent. Reverse transcriptions for the first-strand cDNA synthesis were performed using 5 μg of total RNA as the templates. The equal amounts of the first-strand cDNAs were used for semi-quantitative PCR. The primers for *BoTMT* mRNA were RT6 and RT3. PCR amplifications were conducted under the following conditions: 94 ℃ 4 min; 94 ℃ 8 s, 55 ℃ 8 s, 72 ℃ 8 s, 30 cycles; 72 ℃ 10 min. The primers for *actin1* mRNA were Bact5 (5′-CTGGTTTCGCCGGTGATGATGC-3′) and Bact3 (5′-ATTTCCCGCTCGGCTGTGGTG-3′).

1.4 Construction of the expression vector for *BoTMT*

The cDNA fragment encoding the mature γ-TMT was amplified by RT-PCR with the primers RT5 (5′-CGGGATCCACCATGACAACGACGGCAAC-3′, with a *Bam*H I site) and RT3. The product was cloned into the pGEM-T Easy vector to generate pTMT. The pTMT was digested with *Bam*H I and *Sal* I. A 974 bp DNA fragment was purified and cloned into the *Bam*H I / *Sal* I digested pET30a to obtain the expression vector pET-TMT. pET-TMT was then transformed into *E. coli* BL21 (DE3).

1.5 Expression of *BoTMT* in *E. coli*

The transformants harboring plasmid pET-TMT were cultured at 37 ℃ in LB medium until $OD_{600\,nm}$ reached 0.5～0.7. IPTG was added to a final concentration of 0.4 mmol/L and the cultivation was continued for another 4～5 hours at 37 ℃. The cells were harvested by centrifugation and lysed by sonication. The protein concentration was measured by Bio-Rad Protein Assay. The total bacterial protein was run on 12% SDS-PAGE. The amount of the expression product was analyzed by Imagemaster 1.0 software (Pharmacia).

1.6 The enzyme activity assay of the recombinant γ-TMT

Protein expression was induced as described above except that the growth temperature was 28 ℃. Extracts from induced cells carrying pET-TMT and pET30a plasmids respectively were prepared by homogenizing cell pellets in 10 mmol/L HEPES (pH 7.8), 5 mmol/L DTT, 0.24 mol/L sorbitol,

1 mmol/L PMSF. Triton X-100 was added to a final concentration of 1%, and the homogenates were incubated on ice for 30 min and centrifuged at 30000 g for 30 min at 4 ℃. Protein concentration of supernatant was determined by Bio-Rad Protein Assay, and 60 μL of the supernatant was used for γ-TMT activity analysis.

The γ-TMT activity assay was performed as described by Shintani et al.[14] except that the final concentrations of γ-tocopherol and S-SAM were 0.64 mmol/L and 1.28 mmol/L, respectively. The reaction products were fractionated on Silica GF254 TLC plates in dichloromethane. Tocopherols were identified by comigration with authentic tocopherol standards after observation under UV light (253 nm). For TLC-chromometry assay, a 20 μL aliquot of each reaction was transferred to a new microcentrifuge tube respectively after 0, 20, 40, 60, 80, 100, 120 min incubation. The reaction products were separated on Silica GF254 TLC plates. The bands corresponding to α-tocopherol were scraped from the TLC plates. The A_{520} values were measured in Eemmerie-Engels solution (0.25% α, α′-dipyridyl, 0.1% $FeCl_3$ in ethanol).

2 Results and discussion

2.1 Cloning and analysis of *BoTMT*

Based on the conserved S-SAM binding domain sequences of *Arabidopsis* and *Synechocystis* γ-TMTs, a set of 3′ RACE primers was designed. A 583 bp fragment was obtained by nested PCR and sequenced. Sequence analysis revealed that the cDNA fragment has 82.9% and 44.8% similarity to *Arabidopsis* and *Synechocystis* γ-TMT genes, respectively. The deduced amino acid sequence contains the second SAM binding domain sequence. Therefore, we presumed that the 583 bp fragment was the 3′-end of *B. oleracea* γ-TMT cDNA.

To obtain the 5′-end cDNA fragment, two reverse primers GSP1 and GSP3 were designed according to the 5′-end region of the 583 bp cDNA fragment. A product of 800 bp was obtained by 5′RACE. This fragment shares sequence similarity to *Arabidopsis* and *Synechocystis γ-TMTs* and was considered to be the 5′-end sequence of *B. oleracea* γ-TMT cDNA.

The sequences of the 583 bp and 800 bp fragments were spliced together to form the full-length cDNA of *B. oleracea* γ-TMT. To confirm the whole sequence, RT-PCR was conducted to amplify the coding region sequence with primers RT6 and RT3. A single product was obtained. The nucleic acid sequence of the product is the same as predicted, which demonstrated that the sequence splicing is correct.

The full-length cDNA obtained (GenBank accession number: AF381248) is 1265 bp (Fig. 1) containing an open reading frame of 1044 bp with a 5′ UTR of 26 bp and a 3′ UTR of 195 bp. A putative polyadenylation signal "AATAAA" is located at the down-stream of the sequence, about 13 bp apart from the termination codon "TAA". The complete open reading frame encodes a protein of 347 amino acids with a molecular weight of 38.1 kD. A predicted 47-amino acid chloroplast transit peptide and two conserved SAM binding domains ("XXDXGCGIG", "VXXPGGRXIX") essential to substrate binding are also present in the deduced protein, which is consistent with the structure features common to the known γ-TMTs. Hence, the full-length cDNA named *BoTMT* should be a new member of *γ-TMT* family.

To further analyze the functional and conserved domains of BoTMT, an alignment including γ-TMT sequences from *Arabidopsis*, *Perilla frutescens*, *Sorghum bicolor*, *Nostoc* PCC7120 and *Synechocystis* PCC6803 was performed with GenBank Blast program. The result shows that the similarities of BoTMT with the above γ-TMTs are 86.5%, 71%, 62%, 48%, and 41.8%, respectively. The difference among these γ-TMTs exists mainly in the N- and C-terminal regions, especially in the N-terminal plastidial transit peptide region, whereas the middle functional region is highly conservative across phyla. These data suggest that the γ-TMTs may form similar three-dimension structure though they have low similarities.

2.2 Expression of *BoTMT* in different organs of *B. oleracea*

A semi-quantitative RT-PCR was performed to analyze the *BoTMT* expression level in different organs of *B. oleracea*. The *actin1* gene which has been shown to express constitutively in all organs of *B. oleracea* was used as an internal control. As shown in Fig. 3, *BoTMT* gene was expressed mainly in flowers and leaves of *B. oleracea*, a pattern consistent with the distribution of plastids. The result was confirmed by several independent experiments. It

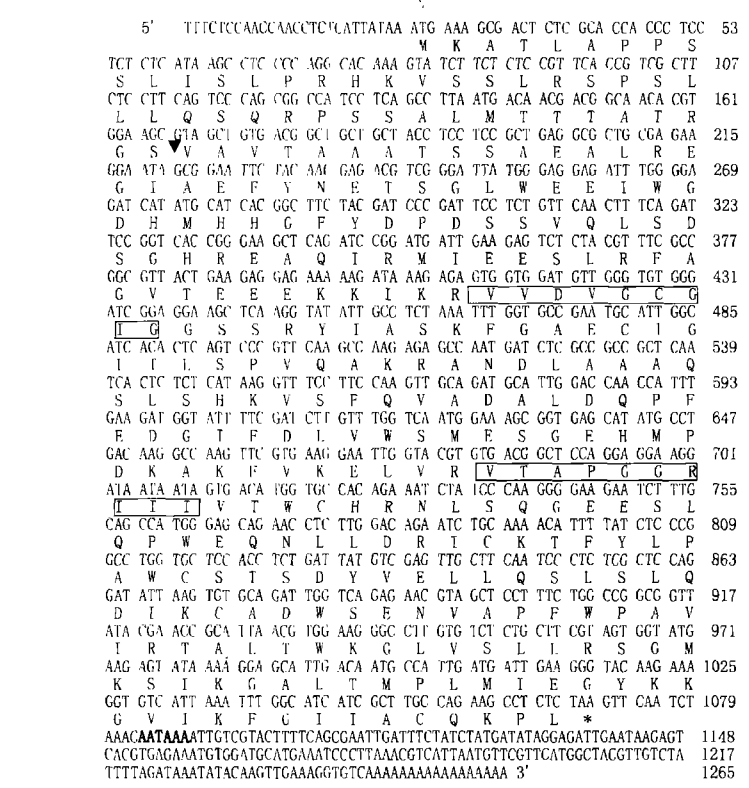

Fig. 1. Nucleotide sequence and deduced amino acid sequence of *BoTMT*. The predicted amino acid sequence of *BoTMT* is shown below the DNA sequence. Predicted cleavage site of N-terminal chloroplast targeting domain is indicated by arrow; two motifs corresponding to conserved SAM binding domains are boxed; a polyadenylation signal is in boldface.

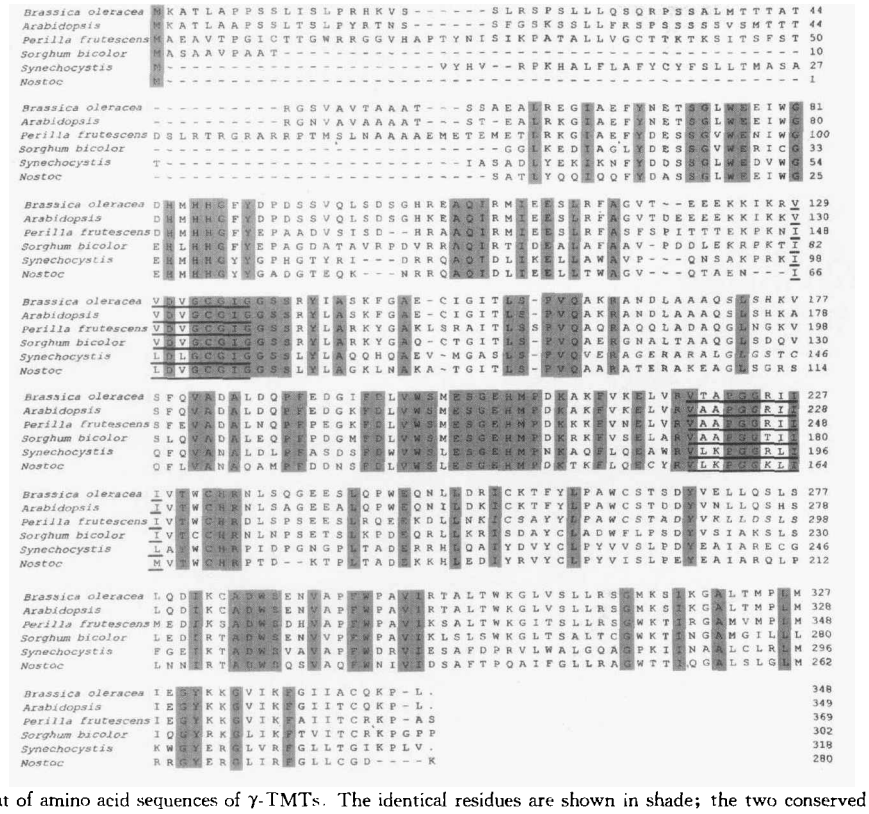

Fig. 2. Alignment of amino acid sequences of γ-TMTs. The identical residues are shown in shade; the two conserved SAM motifs are underlined.

is known that plastids are the biosynthetic sites of α-tocopherol in plant cells[8,15]. In plant photosynthetic tissues (such as green leafy tissues), α-tocopherol is often the most abundant tocopherol although such tissues contain relatively low concentrations of total tocopherols (i. e. between 10 and 50 μg/g fresh weight). Unlike photosynthetic tissues, tissues with less plastids distribution (such as seeds) are often more concentrated in total tocopherols, with their corresponding oils generally containing from 500 to 2000 μg/g tocopherols, of which only a small fraction is α-tocopherol[7]. Our result showed that the expression level of *BoTMT* in plant is consistent with the α- to total tocopherols ratios in different plant tissues, suggesting γ-TMT is likely to have the greatest impact on the level of α-tocopherol accumulated in tissues.

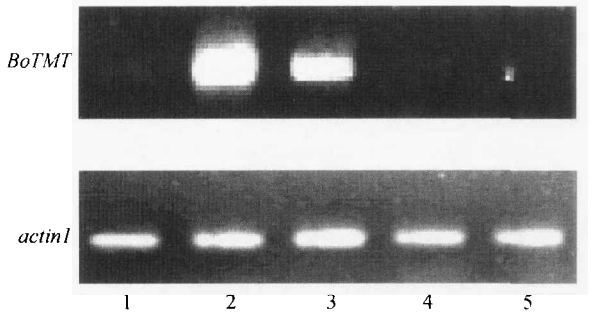

Fig. 3. Semi-quantitative RT-PCR analysis of *BoTMT* expression in different organs of *B. oleracea*. 1, seed; 2, flower; 3, leaf; 4, stem; 5, root.

2.3 Expression of γ-TMT in *E. coli*

The deduced amino acid sequence of *BoTMT* 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 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 946 bp sequence was modified by PCR using a pair of primers RT5 and RT3 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 *BoTMT*, the prokaryotic expression vector pET-TMT was constructed and transformed into *E. coli* BL21 (DE3). After induction at 37 ℃ for 4 h with 0.4 mmol/L IPTG, a 39 kD specific protein band was observed in 12% SDS-PAGE, which had the same molecular weight of the recombinant protein with a 6× His tag sequence of pET30a (Fig. 4, lane 3), while the negative control did not produce this band (Fig. 4, lane 4). The yield of the recombinant γ-TMT protein accounted for 22% of total bacterial protein after inducing for 4 h. The result of ultrasonic treatment showed that a part of the recombinant protein induced at 37 ℃ was soluble (Fig. 4, lane 2), while most are insoluble inclusion bodies (Fig. 4, lane 1).

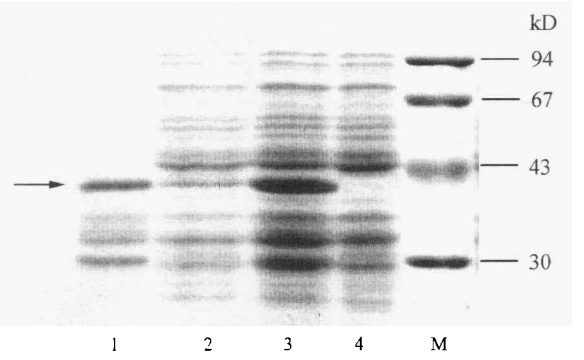

Fig. 4. Expression of the recombinant γ-TMT in *E. coli* induced by IPTG (12% SDS-PAGE). M, molecular marker, the sizes of marker are shown on the right; 1, pellet of *E. coli* BL21 (DE3)/pET-TMT after sonication; 2, supernatant of *E. coli* BL21 (DE3)/pET-TMT after sonication; 3, total protein extracted from *E. coli* BL21 (DE3)/pET-TMT; 4, total protein extracted from *E. coli* BL21 (DE3)/pET30a; the arrow indicates the expressed specific protein.

In order to increase the level of soluble γ-TMT protein for the functional analysis, further studies showed that when induced at 28 ℃, the amount of soluble protein can be increased up to 45% of the total recombinant protein.

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

The recombinant γ-TMT protein from *E. coli* BL21 (DE3)/ pET-TMT was added into a reaction system containing γ-tocopherol and S-SAM. The reaction products were analyzed by fluorescent thin-layer chromatography (Fig. 5). The result showed that a clear fluorescent spot corresponding to authentic α-tocopherol standard appeared in the TLC plate after incubating at room temperature for 1.5 h (Fig. 5, lane 3), while the negative control (*E. coli* BL21 (DE3)/pET30a) has not the corresponding spot (Fig. 5, lane 2). The absorption curve at 520 nm (Fig. 6) also indicated that the value of A_{520} increased with the reaction time in the system containing the

recombinant γ-TMT protein. The value increased significantly in the first 60 min and reached the maximum at 80 min, after that, it decreased slowly. The γ-TMT activity in the reaction system with the recombinant protein was tenfold higher than the negative control at 80 min. These results indicate that the γ-TMT protein expressed in *E. coli* has relatively high enzyme activity to catalyze the methylation of γ-tocopherol to form α-tocopherol.

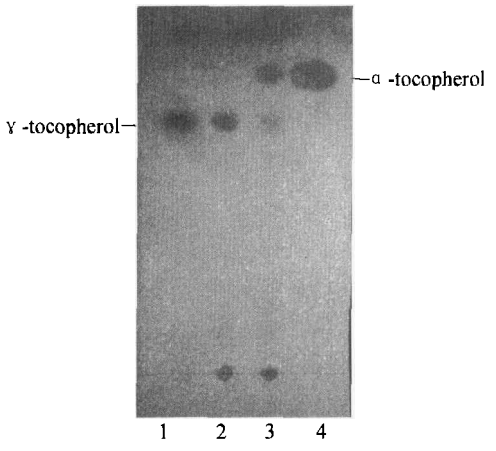

Fig. 5. The activity assay of γ-TMT by TLC. 1, γ-tocopherol standard; 2, the reaction product of negative control pET30a; 3, the reaction product of recombinant γ-TMT; 4, α-tocopherol standard.

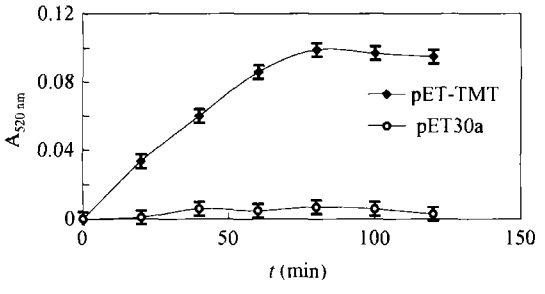

Fig. 6. Changes in absorbance at 520 nm in enzyme activity assay. The value of each point is the average of three repeated experimental data.

In this paper, *B. oleracea* γ-TMT gene, *BoTMT*, has been cloned successfully. Semi-quantitative RT-PCR showed that *BoTMT* is expressed preferentially in flowers and leaves, suggesting the expression level of γ-TMT is responsible for the different proportion of α-tocopherol synthesized and accumulated in plant organs. Overexpression of *BoTMT* has been achieved in *E. coli* and the recombinant γ-TMT has the capability to catalyze the methylation of carbon 5 of the tocopherol chromanol ring, thus to produce α-tocopherol. The recombinant γ-TMT will be useful for the large-scale catalysis *in vitro*.

Our result lays a foundation to further study the role of γ-TMT in tocopherol biosynthesis, and can be applied to elevate the levels of this important antioxidant/vitamin in the major oilseed crops in the future.

References

1 Brigelius-Floh, E. R. et al. Vitamin E: Function and metabolism. The FASEB Journal, 1999, 13: 1145.
2 National Research Council (U. S.). Food and Nutrition Board. NAS-NRC Recommended Dietary Allowances, 10th ed. Washington DC: National Academy Press, 1989, 284.
3 Buring, J. E. et al. Antioxidant vitamins and cardiovascular disease. Nutr. Rev., 1997, 55: S53.
4 Traber, M. G. et al. Vitamin E in humans: demand and delivery. Annu. Rev. Nutr., 1996, 16: 321.
5 Huang, X. S. Natural vitamin E and human health. Liang Shi Yu You Zhi (in Chinese), 2001, 9: 35.
6 Kamal-Eldin, A. et al. The chemistry and antioxidant properties of tocopherols and tocotrienols. Lipids, 1996, 31: 671.
7 Michael, A. et al. Improving the nutrient composition of plants to enhance human nutrition and health. Annu. Rev. Plant Physiol. Plant Mol. Biol., 1999, 50: 133.
8 Soll, J. et al. Localization and synthesis of prenylquinones in isolated outer and inner envelope membranes from spinach chloroplasts. Archives of Biochemistry and Biophysics, 1985, 238(1): 290.
9 d'Garkubgye, A. et al. Plastid enzymes of Terpenoid Biosynthesis: purification and characterization of γ-tocopherol methyltransferase from Capsicum chromoplasts. The Journal of Biological Chemistry, 1985, 260(28): 15200.
10 Koch, M. et al. Characterization of gamma-tocopherol methyltransferases from Capsium annuum L and Arabidopsis thaliana. Eur. J. Biochem., 2003, 270(1): 84.
11 Ishiko, H. et al. Some properties of γ-tocopherol methyltransferase solubilized from spinach chloroplasts. Phytochemistry, 1992, 31(5): 1499.
12 Shigeoka, S. et al. Isolation and properties of γ-tocopherol methyltransferase in Euglena gracilis. Biochimica et Biophysica Acta, 1992, 1128: 220.
13 Michalowski, M. et al. Preliminary charaterization of S-adenosylmethionine: tocopherol methyltransferase from chloroplasts of Calendula officinalis seedlings. Acta Biochim. Pol., 1993, 40(1): 116.
14 Shintani, D. et al. Elevating the vitamin E content of plants through metabolic engineering. Science, 1998, 282: 2098.
15 Soll, J. et al. Site of Biosynthesis of alpha-tocopherol in spinach chloroplasts. FEBS letters, 1980, 112(2): 243.