BIOLOGIA PLANTARUM 56 (1): 131-134, 2012

BRIEF COMMUNICATION

# Expression of γ-tocopherol methyltransferase gene from *Brassica napus* increased α-tocopherol content in soybean seed

D.F. CHEN, M. ZHANG, Y.Q. WANG and X.W. CHEN*

*Laboratory of Molecular Genetics, College of Life Sciences, Nankai University, Tianjin 300071, P.R. China*

## Abstract

A cDNA encoding γ-tocopherol methyltransferase from *Brassica napus* (*BnTMT*) was overexpressed in soybean [*Glycine max* (L.) Merr.] under the control of seed-specific promoter of *Arabidopsis* fatty acid elongase 1 (FAE1) or soybean glycinin G1. Two and three transgenic plants were selected, respectively, after *Agrobacterium*-mediated transformation. Polymerase chain reaction (PCR) and Southern blots confirmed that *BnTMT* was single-copy integrated into the genome of transgenic plants. RT-PCR analysis showed that the expression of *BnTMT* was higher in the immature cotyledons than in the mature cotyledons, while no expression was detected in the leaves. Moreover, the expression level under the control of FAE1 was higher than that of G1. HPLC analysis indicated that the seed-specific expression of *BnTMT* resulted in 11.1-fold and 18.9-fold increase in α- and β-tocopherol content, respectively, in $T_2$ seed. These results suggested that introducing *BnTMT* into soybean can be used to increase the vitamin E composition in seeds.

*Additional key words*: fatty acid elongase 1, *Glycine max,* glycinin, RT-PCR, Southern blot, transgenic plants.

Tocopherols are synthesized exclusively by photosynthetic organisms. Usually, α-tocopherol is the predominant form found in leaves, while γ-tocopherol and tocotrienols accumulate to higher levels in seed of many plant species (Tan 1989, Demurin *et al*. 1996). Oilseeds are particularly rich in tocopherols with an average concentration 10-fold higher than other plant tissues. However, soybean seed generally contains relatively high content of γ-tocopherol (60 - 65 % of the total) and δ-tocopherol (20 - 26 %) which has lower vitamin E activity, but very low α-tocopherol (7 - 10 %) which has higher vitamin E activity (Shintani and DellaPenna 1998, Grusak 1999). Therefore, conversion of γ-tocopherol to α-tocopherol in soybean seed could increase its nutritional value and significance in human health.

Gamma-tocopherol methyltransferase (γ-TMT) is a rate-limiting enzyme in the synthesis of α- and β-tocopherols from γ- and δ-tocopherols, respectively (Hofius and Sonnewald 2003, Koch *et al*. 2003).Therefore, seed specific expression of γ-TMT gene could be a way to improve the vitamin E composition in soybean seed. Genes encoding γ-TMT have been isolated from *Arabidopsis thaliana* (Shintani and DellaPenna 1998) and *Perilla frutescens* (Tavva *et al*. 2007). Transgenic *Arabidopsis* (Shintani and DellaPenna 1998), lettuce (Van Eenennaam *et al*. 2003, Li *et al*. 2011), soybean (Van Eenennaam *et al*. 2003, Tavva *et al*. 2007), *Brassica juncea* (Yusuf and Sarin 2007), perilla (Lee *et al*. 2008) and *Codonopsis lanceolata* (Seong *et al*. 2009) with high α-tocopherol content in their seeds or leaves have also been developed by overexpressing these two genes.

We have previously isolated a cDNA encoding γ-tocopherol methyltransferase from *Brassica napus* (*BnTMT*, GenBank number DQ508019) and its expression product in *Escherichia coli* was shown to have the activity to catalyze transformation of γ-tocopherol into α-tocopherol (Qian *et al*. 2007). The objective of this study was to investigate whether introducing *BnTMT* into soybean under the control of seed-specific

*Received* 2 April 2010, *accepted* 7 September 2010.

*Abbreviations*: *BnTMT* - γ-tocopherol methyltransferase gene from *Brassica napus*; FAE1 - fatty acid elongase 1; PCR - polymerase chain reaction; RT-PCR - real time PCR.

*Acknowledgements*: The research was supported by the key program of the Natural Science Foundation of Tianjin (grant No. 07JCZDJC03800), the grant of the National Natural Science Foundation of China (No. 31070273), and the R & D Special Projects for Public Welfare Industry of State Oceanic Administration People's Republic of China (No. 200805044).

* Corresponding author; fax: (+86) 22 23500133, e-mail: xiwenchen@nankai.edu.cn

promoter of *Arabidopsis* fatty acid elongase 1 (FAE1) or soybean glycinin G1 could improve *α*-tocopherol content in soybean seeds and to compare the effect of two promoters in directing the expression of *BnTMT* in transgenic plants.

Seed specific promoters of FAE1 (GenBank No. AF355982) and G1 (GenBank No. DQ250808) were isolated using PCR with leaf DNA from *Arabidopsis* or soybean as template, FAE1-forward (F) (5′-CCCC AAGCTTTAGCCTAAGTACGTACTCAAAATGCC-3′, *Hin*dIII site was introduced) and FAE1-reverse (R) (5′-AAAACTGCAGGGTGATGACTGATGTGTTAAGG-3′, *Pst*I site was introduced), and G1-F (5′-CCCCAAGCTT CTAGTAGATTGGTTGGTTGGTTTCCC-3′, *Hin*dIII site was introduced) and G1-R (5′-AAAACTGCAG TGCTCTGTTTGTGTCGGAAAATAAT-3′, *Pst*I site was introduced) as primers according to the respective references (Maren *et al*. 2001, Ding *et al*. 2006). A 934 bp FAE1 and a 688 bp G1 fragment were amplified and then cloned into pMD-18T vector (*TaKaRa*, Dalian, China) for sequencing. After confirming the accuracy of their expression elements, they were ligated with *BnTMT* (Qian *et al*. 2007) separately and then inserted into the *Hin*dIII/*Sal*I site of pBin438 binary vector after removing the Ω enhancer and double CaMV35S promoter. The resulted constructs (pFAE1-TMT, pG1-TMT, Fig. 1*A*) were introduced into *Agrobacterium tumefaciens* LBA4404 by freeze-thaw method.

The soybean [*Glycine max* (L.) Merr.] cultivar nf37, which was proven as a good tissue culture responsive genotype (data not shown), was used for the experiment. The embryonic tips were collected from mature seeds and inoculated with *Agrobacterium* suspension following the method described previously (Liu *et al*. 2004). Rooted transgenic $T_0$ plants were grown to maturity in a greenhouse under about 16-h photoperiod under natural irradiance.

Genomic DNA was isolated from kanamycin-resistant plants using CTAB method. The primer pairs specific for detecting *BnTMT*, FAE1 and G1 were TMT-P (5′-AAACTGCAGATGAAAGCGACTCTCGCACC-3′) and TMT-E4 (5′-ACGCGTCGACTTAGAGAGGTTT CTGGCAAGTGATG-3′) (Qian *et al*. 2007), FAE1-F and FAE1-R, G1-F and G1-R, respectively. Seven transformed plants (F7, F21 and F26 carrying pFAE1-TMT, G11, G18, G25 and G35 carrying pG1-TMT) which were positive in PCR analysis for both *BnTMT* and promoter were selected for Southern blot following a procedure of Sambrook and Russel (2001) with minor modifications. Briefly, 10 μg of genomic DNA from each plant was digested overnight with *Hin*dIII, separated on a 0.7 % agarose gel, and transferred to nylon membrane (*Boehringer*, Ingelheim, Germany). *BnTMT* gene, which was amplified using primers TMT-P and TMT-E4 descried before were labeled with *α*-$^{32}$P-dCTP.

Total RNA was isolated from 100 mg of young leaves, immature cotyledons (4 - 7 mm) and mature cotyledons (8 - 10 mm) using *RNAultra* extraction kit (*Qiagen*, Valencia, USA). First strand cDNAs were synthesized from 2.0 μg of total RNA using *AMV* reverase transcriptase (*Promega*, Madison, USA) following the manufacture's protocol. The targeted fragment of *BnTMT* (424 bp) was amplified in 50 mm$^3$ reaction mixture containing 5 mm$^3$ template, 1.25 U *ExTaq* (*TaKaRa*), 1× PCR buffer and 0.2 mM of dNTP each by using TMT-5 (5′-AATGAAAGCGACTCTCGCACC-3′) and TMT-6 (5′-CCTTGAGCTTCCTCCGATCC-3′) as primers. A 225 bp fragment of soybean actin was also amplified, as the quantitative control of RT-PCR reaction, under the same conditions with actin-R (5′-TTAGAAGCA CTTGCGGTGCACG-3′) and actin-F (5′-GTACTGCAA CATCGTGCTGTCG-3′) (Zeng *et al*. 2006) as primers.

Mature seeds collected from $T_2$ transgenic plants were

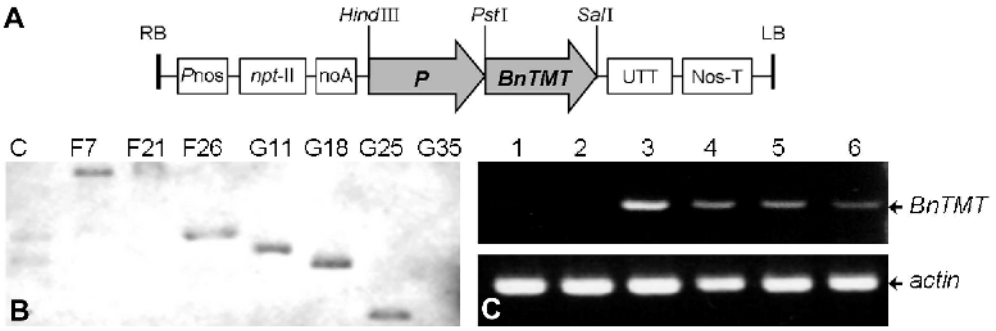


Fig. 1. Analyses of transgenic soybean plants for *BnTMT* integration and expression. *A* - Schematic representation of the seed-specific expression vector pFAE1-TMT or pG1-TMT: *P* - FAE1 or G1 promoter, *BnTMT* - γ-tocopherol methyltransferase gene from *Brassica napus*, UTT and Nos-T - termination sequence of transcription, *npt*-II - neomycin phosphatransferase II. *B* - Southern blot analysis of genomic DNA digested with *Hin*dIII. F7, F21, F26, G11, G18, G25 and G35 - the $T_0$ transformants, which were positive in both transgene and promoter PCR analysis. F7 F21 and F26 are the plants carrying pFAE1-TMT, while G11, G18, G25 and G35 are the plants carrying pG1-TMT; C - untransformed control. *C* - Expression pattern of *BnTMT* in transgenic $T_1$ lines using semi-quantitive PCR; *upper panel* - the expression of *BnTMT lane*s 1, 3 and 5 were leaf, immature cotyledon and mature cotyledon of F7, *lanes* 2, 4 and 6 were leaf, immature and mature seed of G18; *lower panel* - the expression of *actin* as the quantitative control. Samples collected from 15 pods of at least three progenies which were PCR-positive of each line. Three replicates were performed.

Table 1. The content and composition of tocopherols in the seeds of $T_2$ transgenic soybean under the control of FAE1 and G1. F7 and F26 are the lines carrying pFAE1-TMT while G11, G18 and G25 are the lines carrying pG1-TMT. Samples were collected from 15 pods of at least three plants of each line. Means ± SE.

| Line | Total tocopherols [μg $g^{-1}$(d.m.)] | α-Tocopherol [μg $g^{-1}$(d.m.)] | [%] | β-Tocopherol [μg $g^{-1}$(d.m.)] | [%] | γ-Tocopherol [μg $g^{-1}$(d.m.)] | [%] | δ-Tocopherol [μg $g^{-1}$(d.m.)] | [%] |
|---|---|---|---|---|---|---|---|---|---|
| Control | 365.7 ± 10.2 | 30.7 ± 2.8 | 8.4 | 2.9 ± 0.1 | 0.8 | 267.7 ± 3.2 | 73.2 | 64.4 ± 1.1 | 17.6 |
| F7 | 378.2 ± 13.5 | 340.0 ± 11.6 | 89.9 | 38.2 ± 5.2 | 10.1 | 0.0 ± 0.0 | 0.0 | 0.0 ± 0.0 | 0.0 |
| F26 | 386.5 ± 11.8 | 330.8 ± 12.2 | 85.6 | 54.9 ± 4.6 | 14.2 | 0.8 ± 0.1 | 0.2 | 0.0 ± 0.0 | 0.0 |
| G11 | 375.6 ± 16.5 | 294.8 ± 11.7 | 78.5 | 36.8 ± 3.2 | 9.8 | 34.6 ± 3.3 | 9.2 | 9.4 ± 0.6 | 2.5 |
| G18 | 367.4 ± 12.3 | 291.0 ± 9.9 | 79.2 | 32.0 ± 2.2 | 8.7 | 32.3 ± 3.1 | 8.8 | 12.1 ± 0.9 | 3.3 |
| G25 | 381.2 ± 15.6 | 309.9 ± 14.7 | 81.3 | 31.3 ± 2.5 | 8.2 | 29.0 ± 2.2 | 7.6 | 11.1 ± 0.5 | 2.9 |

used for tocopherol analysis using a method of Savidge *et al.* (2002) with minor modification. Approximately 500 mg of seeds was grounded in liquid nitrogen, transferred to 2 $cm^3$ of 0.8 % (m/v) butylated hydroxytoluene in ethanol, and then filtered through a 0.2 m filter. A 0.75 $cm^3$ aliquot of filtrate was then dried under nitrogen gas, resuspended in 0.75 $cm^3$ of hexane and then subjected to HPLC analysis (*Agilent 1100*, Wilmington, USA) on a *Venusil* silica 5 μm, 250 mm × 4.6 mm normal phase column (*Agela Technologies*, Wilmington, USA) at 42 ºC with a flow rate of 2 $cm^3$ $min^{-1}$ with 83 % (v/v) hexane and 17 % (v/v) isopropyl ether. Tocopherols were detected by fluorescence using 290 nm excitation and 325 nm emission.

A total of 29 and 36 kanamycin-resistant soybean plants potentially carrying pFAE1-TMT and pG1-TMT were obtained respectively. PCR analysis, performed using primer specific for transgene, indicated that 6 of 29 (20.7 %) and 7 of 36 (19.4 %) plants carrying pFAE1-TMT and pG1-TMT contained *BnTMT*, respectively. PCR analysis, performed using primer specific for each promoter, indicated that 3 of 6 (50 %) and 4 of 7 (57.1 %) of plants carrying pFAE1-TMT or pG1-TMT contained the specific promoter sequence, respectively. Those plants (F7, F21, F26, G11, G18, G25 and G35), which were positive in both transgene and promoter analysis, were used for Southern analysis. As shown in Fig. 1*B*, one band appeared in 2 of the pFAE1-TMT plants and 3 of the pG1-TMT plants, indicating that one-copy of *BnTMT* gene was integrated into the genome of these five transgenic soybean plants.

Seeds of these transgenic $T_0$ plants were obtained by self-pollination. Compared with nontransformed control plants, number of flowers and seeds set in transgenic plants were 10 to 20 % lower than in the control plants. Germination of $T_0$ seeds was similar to that of control seeds (data not shown). After preliminary PCR analysis, semi-quantitive RT-PCR analysis was performed to reveal the expression pattern of *BnTMT* in the PCR-positive transgenic $T_1$ lines. All the five transgenic lines had higher level of *BnTMT* expression in the immature cotyledons than in the mature cotyledons, while no expression was detected in the leaves (Fig. 1*C*), which confirmed the seed-specific expression pattern of *BnTMT*. Moreover, the expression level under the control of FAE1 is higher than that under of G1 at each developmental stage indicating the higher activity of FAE1 compared to G1 in directing *BnTMT* expression.

The content and composition of tocopherols in transgenic $T_2$ plants was analyzed by HPLC analysis (Table 1). In the wild type seeds, most of the tocopherol existed as γ-tocopherol (73.2 %). Whereas, in the seeds of five transgenic lines, 64.0 - 73.2 % γ-tocopherol and 14.3 - 17.6 % δ-tocopherol in the seeds was converted into α-tocopherol and β-tocopherol, respectivley. The conversion was more complete under the control of FAE1 with only one plant line leaving 0.2 % γ-tocopherol in the seed, while the three transgenic lines under the control of G1 still retained 7.6 - 9.2 % γ-tocopherol and 2.5 - 3.3 % δ-tocopherol.

Increasing vitamin E activity by overexpressing γ-TMT gene in soybean seed could enhance the nutritional values of the crop. Although there are several sequences of the genes stored in GeneBank, only the genes from *Arabidopsis* (Shinatani and DellaPenna 1998, Cho *et al*. 2005, Yusuf and Sarin 2007) and *Perilla* (Tavva *et al*. 2007, Lee *et al*. 2008) has been introduced into plants and caused an increase of *α*-tocopherol content in their seeds or leaves. Our data showed that *BnTMT* could increase *α*-tocopherol content in the soybean seeds. *B. napus* was chosen as the gene donor because it is also an oilseed crop, and its genes may express in soybean without the potential silencing problems which arises from overexpressing the endogenous soybean genes.

The expression of a heterogonous gene in plant cell is influenced by a number of factors. Among them, the promoter plays a central role. In this study we have shown that *BnTMT* overexpressed in soybean under the control of seed-specific promoters FAE1 or G1 increased the *α*-tocopherol content significantly in the $T_2$ transgenic soybean seed. FAE1 promoter was more efficient than G1 for the conversion, which was consistent with the higher expression level of *BnTMT* in the transgenic soybeans. Therefore, the higher expression of *BnTMT* in the transgenic soybeans carrying pFAE1-TMT may be due to the higher activity of FAE1. The *in vivo* activity of FAE1 was also reported to be superior to napin promoter (Maren *et al*. 2001). FAE1 could also direct the gene

expression in the early torpedo stage embryos 4 - 5 d after flowering and was proposed as a suitable promoter for seed oil engineering (Maren *et al*. 2001). Although the lack of interplay between the fatty acid and tocopherol biosynthesis was suggested (Lee *et al*. 2008), the role of *α*-tocopherol in preventing oxidative damage to lipid components may make the two biosynthesis processes linked. Further research is needed to investigate whether FAE1 is also a suitable promoter for vitamin E engineering.

In conclusion, five transgenic soybean lines over-expressing *BnTMT* under the control of seed-specific promoters FAE1 or G1 resulted in nearly complete conversion of all tocopherols present in the seed to α- and β-tocopherols in their seeds. These results suggested that introducing *BnTMT* into soybean could express functional enzyme and can be used to improve the vitamin E composition through transgenic approach.

## References


Cho, E.A., Lee, C.A., Kim, Y.S., Back, S.H., de los Reyes, B.G., Yun, S.J.: Expression of *γ*-tocopherol methyltransferase transgene improves tocopherol composition in lettuce (*Latuca sativa* L.). - Mol. Cells **19**: 16-22, 2005.

Demurin, Y., Skoric, D., Karlovic, D.: Genetic variability of tocopherol composition in sunflower seeds as a basis of breeding for improved oil quality. - Plant Breed. **115**: 33-36, 1996.

Ding, S.H., Huang, L.Y., Wang, Y.D., Sun, H.C., Xiang, Z.H.: High-level expression of basic fibroblast growth factor in transgenic soybean seeds and characterization of its biological activity. - Biotechnol Lett. **28**: 869-875, 2006.

Grusak, M.A., Della Penna, D.: Improving the nutrient composition of plants to enhance human nutrition and health. - Annu. Rev. Plant Physiol. Plant mol. Biol. **50**: 133-161, 1999.

Hofius, D., Sonnewald, U.: Vitamin E biosynthesis: biochemistry meets cell biology. - Trends Plant Sci. **8**: 6-8, 2003.

Koch, M., Lemke, R., Heise, K.P., Mock, H.P.: Characterization of gamma-tocopherol methyltransferases from *Capsicum annuum* L and *Arabidopsis thaliana*. - Eur. J. Biochem. **270**: 84-92, 2003.

Lee, B.K., Kim, S.L., Kim, K.H., Yu, S.H., Lee, S.C., Zhang, Z.Y., Kim, M.S., Park, H.M., Lee, J.Y.: Seed specific expression of perilla *γ*-tocopherol methyltransferase gene increases *α*-tocopherol content in transgenic perilla (*Perilla frutescens*). - Plant Cell Tissue Organ Cult. **92**: 47-54, 2008.

Li, Y., Wang, G., Hou, R., Zhou, Y., Gong, R., Sun, X., Tang, K.: Engineering tocopherol biosynthesis pathway in lettuce. - Biol. Plant. **55**: 453-460, 2011.

Liu, H.K., Yang, C., Wei, Z.M.: Efficient *Agrobacterium tumefaciens*- mediated transformation of soybeans using an embryonic tip regeneration system. - Planta **219**: 1042-1049, 2004.

Maren, R., Mark, S., Ljerka, K.: Expression of the FAE1 gene and FAE1 promoter activity in developing seeds of *Arabidopsis thaliana*. - Plant mol. Biol. **46**: 717-725, 2001.

Qian, W.C., Chen, D.F., Wang, H.Z., Wang, Y.Q., Chen, X.W.: [Gene cloning and function confirmation of *γ*-tocopherol methyltransferase from *Brassica napus* L.] - Plant Physiol. Commun. **43**: 68-688, 2007. [In Chin.]

Sambrook, J., Russel, D.W. (ed.): Molecular Cloning: a Laboratory Manual. 3rd Ed. - Cold Spring Harbor Laboratory Press, Cold Spring Harbor 2001.

Savidge, B., Weiss, J.D., Wong, Y.H., Lassner, M.W., Mitsky, T.A., Shewmaker, C.K., Post-Beittenmiller, D., Valentin, H.E.: Isolation and characterization of homogentisate phytyltransferase genes from *Synechocystis* sp. PCC 6803 and *Arabidopsis*. - Plant Physiol. **129**: 321-332, 2002.

Shinatani, D., Della Penna, D.: Elevating the vitamin E content of plants through metabolic engineering. - Science **282**: 2098-2100, 1998.

Seong, E.S., Ghimire, B.K., Goh, E.J., Lim, J.D., Kim, M.J., Chung, I.M., Yu, C.Y.: Overexpression of the γ-TMT gene in *Codonopsis lanceolata*. - Biol. Plant. **53**: 631-636, 2009.

Tan, B.: Palm carotenoids, tocopherols and tocotrienols. - J.amer. oil. chem. Soc. **66**: 770-776, 1989.

Tavva, V.S., Kim, Y.H., Kagan, I.A., Dinkins, R.D., Kim, K.H., Collins, G.B.: Increased *α*-tocopherol content in soybean seed overexpressing the *Perilla frutescens* *γ*-tocopherol methyltransferase gene. - Plant Cell Rep. **26**: 61-70, 2007.

Van Eenennaam, A.L., Lincoln, K., Durrett, T.P., Valentin, H.E., Shewmaker, C.K., Thorne, G.M., Jiang, J., Baszis, S.R., Levering, C.K., Aasen E.D., Hao, M., Stein, J.C., Norris, S.R., Last, R.L.: Engineering vitamin E content: from *Arabidopsis* mutant to soy oil. - Plant Cell **15**: 3007-3019, 2003.

Yusuf, M.A., Sarin, N.B.: Antioxidant value addition in human diets: genetic transformation of *Brassica juncea* with *γ*-TMT gene for increased *α*-tocopherol content. - Transgenic Res. **16**: 109-113, 2007.

Zeng, J., Wang, Y.C., Shen, G., Zheng X.B.: [A *Phytophthora sojae* gene of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) induced in host infection and its anti-oxidative function in yeast.] - Chin. Sci. Bull. **51**: 1316-1323, 2006. [In Chin.]