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Full Length Research Paper

Molecular cloning, *in silico* chromosome location and expression analysis of gamma-tocopherol methyltransferase gene in common wheat

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Tocopherols, known collectively as vitamin E, are micronutrients with antioxidant properties synthesized only by photosynthetic organisms that play important roles in human and animal nutrition. The enzyme gamma-tocopherol methyltransferase (γ-TMT) converts δ- and γ-tocopherol to β- and α-tocopherol, which is a committed step to elevate vitamin E activity and nutrition value. Here, we presented the cloning, chromosome location and expression characterization of a cDNA from common wheat (*Triticum aestivum* L.) that encoded a putative γ-TMT. This gene (designated *TaTMT*, accession number: DQ139266) had a total length of 1288 bp with an open reading frame of 1098 bp, and encoded a predicted polypeptide of 365 amino acids with a molecular weight of 39.5 kDa and an isoelectric point of 7.2. The deduced amino acid sequence shared high similarity to those of the previously cloned γ-TMT genes from other plants and had a chloroplast transit peptide predicted by TargetP algorithm. Chromosome location of *TaTMT* based on sequence similarity and map position of the EST was mapped in wheat 6AL, 6BL and 6DL chromosomes. In *silico*, expression analysis revealed that *TaTMT* was constitutively expressed in various organs, whereas it was not detectable in callus, cell culture and sheath. Semi-quantitative RT-PCR showed that *TaTMT* was abundantly expressed in stem and leaf, but was showed very low expression level in root, indicating the expression of *TaTMT* was tissue specific in photosynthetic organs. However, the expression level of *TaTMT* was not regulated by high light oxidative stress and this was not surprising because γ-TMT can only convert tocopherol types but not the total volume of antioxidant tocopherols. These molecular analysis results provided fundamental and important information for genetic improvement of bread wheat vitamin E micronutrient quality.

Key words: *Triticum aestivum*, vitamin E, gamma tocopherol methyltransferase, gene cloning, gene expression.

INTRODUCTION

Tocopherols are amphipathic, lipid-soluble antioxidants that are produced by photosynthetic organisms. The natural four tocopherols, α-, β-, γ- and δ-tocopherol, differ only in the number and position of methyl substituent on the aromatic ring (Fryer, 1992). They function as lipid-soluble antioxidants that scavenge reactive oxygen species or singlet oxygen and protect polyunsaturated fatty acids from lipoxygenase attack, especially during seedling germination (Krieger-Liszkay and Trebst, 2006; Sattler et al., 2004, 2006). α-tocopherolquinone was the first reported α-tocopherol oxidation product over 40 years ago in *Spinacia oleracea*, *Euglena gracillis* and *Zea mays* (Dilley and Crane, 1963; Threlfall and Goodwin, 1967; Threlfall et al., 1968), and its levels are correlated with drought, water and high light stress (Munne-Bosch and Alegre, 2003; Kobayashi and DellaPenna, 2008).

Except for acting as antioxidant, tocopherols, known collectively as vitamin E, are also essential micronutrients

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Abbreviations: EST, expressed sequence tag; **γ-TMT,** gamma tocopherol methyltransferase; **TaTMT,** *Triticum aestivum* tocopherol methyltransferase

in human diet required at minimum level to alleviate nutritional disorder. Daily intake of vitamin E in excess of recommended daily allowance (RDA) is associated with decreased risk of cardiovascular disease and some cancers, improved immune function, and slowing of the progression of a number of degenerative human conditions (Traber and Sies, 1996). Although, tocopherols have similar lipid-soluble antioxidant activities *in vitro* and are absorbed equally by human, α-tocopherol has the highest vitamin E activity because of the preferential retention by a hepatic α-tocopherol transfer protein (α-TTP) (Traber and Arai, 1999). Nevertheless, the dietary vitamin intake which is mainly from vegetable oils has relatively low α- to γ-tocopherol ratio (Eitenmiller, 1997; Grusak, 1999).

In the past decade, the tocopherol biosynthetic pathway in photosynthetic organisms has been elucidated (DellaPenna, 2005; DellaPenna and Pogson, 2006). In plants, the committed step in head group synthesis is the *p*-hydroxyphenylpyruvic acid dioxygenase (HPPD) which converts *p*-hydroxyphenylpyruvic acid (HPP) to homogentisic acid (HGA) (Norris et al., 1995, 1998; Schulz et al., 1993). Then, HGA is prenylated with phytyl-DP (diphosphate) to yield the first committed intermediates in tocopherol synthesis catalyzed by homogentisate prenyltransferase (HPT) (Cahoon et al., 2003; Collakova and DellaPenna, 2001; Savidge et al., 2002; Schledz et al., 2001). After that, two tocopherols, δ- and γ-tocopherol, are produced by tocopherol cyclase (Sattler et al., 2003; Porfirova et al., 2002; Provencher et al., 2001). Finally, γ-tocopherol methyltransferase (γ-TMT) adds a methyl group to the aromatic ring converting δ- and γ-tocopherol to β- and α-tocopherol (Shintani and DellaPenna, 1998). Seed-specific overexpression of γ-TMT in *Arabidopsis* resulted in the near-complete conversion of γ-tocopherol to α-tocopherol and a nine fold increase in vitamin E activity (Shintani and DellaPenna, 1998). Same results have also been obtained in soybean when *Arabidopsis* γ-TMT was over expressed in seed-specific manner leading to a nearly fivefold increase in the vitamin activity of soybean oil (Van Eenennaam, 2003). Hence, over expressing the γ-TMT gene in commercially important crops should similarly elevate α-tocopherol levels and thereby increase the vitamin E nutritional value.

Bread wheat (*Triticum aestivum* L.) is one of the world's most important staple crops. Nowadays, genetic improvement of common wheat quality including micronutrients is emerging to a main breeding objective. However, little is known regarding the mechanism of micronutrients genetic control, especially in the vitamin E biosynthesis pathway. The main aims of this study are: (1) cloning and analysis of *TaTMT* gene from common wheat background; (2) locating this gene on chromosome by *in silico* method and (3) detecting its expression profile on different tissues and under light stress. The results will provide fundamental knowledge to elevate vitamin E nutrient quality by genetic engineering.

MATERIALS AND METHODS

Plant material and treatment

Seeds of common wheat (*Triticum aestivum* L.) Jing 411 were surface sterilized with 4% sodium hypochlorite for 30 min and then rinsed with distilled water for 12 h at 37°C. Seedlings were grown in a controlled chamber with a 10 h photoperiod at 75 to 100 µmol photons m^{-2} s^{-1} (25°C/22°C day/night cycle) for 4 weeks. To prevent nutrient depletion, plants were watered with 0.5× Hoagland solution instead of water twice a week. At 4 weeks of age, some plants were transferred to high light (0.8^{-1} mmol photons m^{-2} s^{-1}, 10-h photoperiod) for stress experiments while others maintained at 75 to 100 µmol photons m^{-2} s^{-1}. In order to exacerbate oxidative stress, nutrient limitation was also imposed on plants grown at high light by watering only with distilled water during the course of stress treatment (Collakova and DellaPenna, 2003). Leaves were harvested for RNA extraction at indicated time points within 3 to 5 h after the start of the light cycle.

Ribose nucleic acid (RNA) extraction and cDNA synthesis

Total RNA was extracted from wheat seedlings using TRIZOL reagent and treated with DNase I according to the manufacturer's instructions (GIBCO-BRL, USA). The first-strand cDNA was synthesized by using SuperScript II reverse transcriptase and oligo(dT) primer (TaKaRa, Japan) according to the manufacturer's instructions.

Isolation of the full-length cDNA of *TaTMT*

Full-length cDNA was generated using the rapid amplification of cDNA ends (RACE) system (Invitrogen, Carlsbad, CA). For 5' RACE, first strand cDNA was synthesized according to the protocol of the 5' RACE system using the AP primer (Invitrogen) and was tailed with oligo dC according to the manufacturer's instructions.

Sequence analysis

Sequence analysis was performed using the software DNAMAN (version 3.0, Lynnon BioSoft). BLAST search was conducted in NCBI (http://www.ncbi.nlm.nih.gov) and protein prediction was performed in CBI (http://www.cbi.pku.edu.cn). Chloroplast transit peptide was searched with TargetP 1.1 (http://www.cbs.dtu.dk/services/TargetP/).

In silico expression analysis

Statistical analysis of gene expression profiles was performed using EST data as constituents of UNIGENE. Similarity of gene expression was estimated using Pearson's correlation coefficient, as described by Eisen et al. (1998). The expression profile is displayed based on the number of constituents in a Unigene.

Chromosome location mapping

Over 6596 EST sequences have been mapped in wheat chromosome bins using deletion lines (Qi et al., 2004) (http://www.graingenes.org/cgi-bin/ace/custom/goBLAST). When the sequence has high similarity to one EST in the database, the chromosome location will be defined (Akhunov et al., 2003).

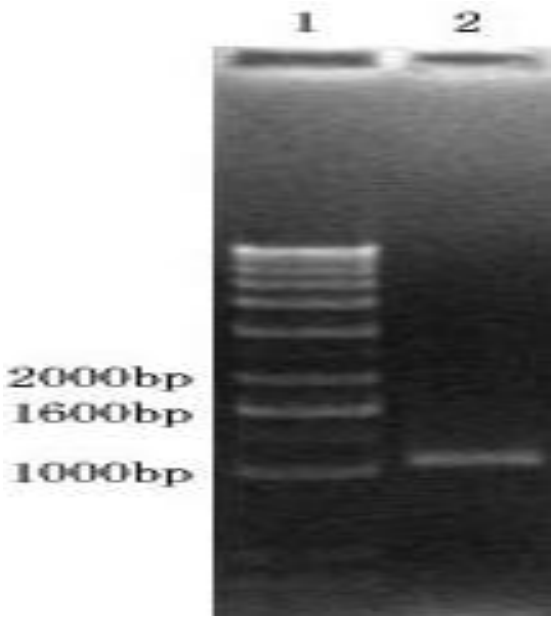

Figure 1. Amplification of TaTMT gene by RT-PCR. 1. DNA ladder; 2. RT-PCR product.

Semiquantitative RT-PCR

A measure of 1μg of total RNA was used to synthesize the first strand of cDNA. Primer pairs of *TaTMT* used in semiquantitative RT-PCR are as follows: forward: 5'-CGAGGCTCCACATACAAA-3', reverse: 5'-CCTCAGGTTCTCCTTCTATG-3'. Two microliter of the first strand cDNA was used as template in 25 μl reaction volume containing 1 × PCR buffer, 1.5 mM of magnesium ion, 200 μM dNTPs, 1.5 units of Taq polymerase (Promega), 40 μM gene specific primers under the following conditions: 95°C for 2min, followed by 35 cycles each of 94°C for 30 s, 55°C for 30 s and 72°C for 60 s. The housekeeping gene 18SrRNA was chosen as internal control and amplified in the same aliquot using specific primers: forward: 5'- TGTGAAACTGCGAATGGC -3' and reverse: 5'- TCGGCATCGTTTATGGTTG -3'. The PCR products (10 μl) were separated on 1% agarose gels stained with ethidium bromide (10 μg/ml). The quantity of the products was analyzed with gene analysis software package (Gene company, USA).

RESULTS AND DISCUSSION

Isolation of wheat gamma-TMT full-length cDNA

Initially, tBLASTn blast search (www.ncbi.nlm.nih.gov) showed that an EST sequence BU101270 shared high sequence homology with *Arabidopsis* γ-TMT, implying that it was probably a part of γ-TMT gene. So, the 5' and 3' sequences of the EST were cloned by RACE. These 5' and 3' end fragments, together with the BU101270 EST sequence, were assembled into a 1288 bp cDNA contig. Finally, amplification of the full-length cDNA was conducted directly from the original RNA sample using the 5' most and 3' most primers of the assembled cDNA contig. To minimize PCR amplification error, a high-fidelity PCR polymerase was employed and the amplification was repeated three times.

The full-length wheat γ-TMT cDNA was 1288 bp which was predicted to have an initiation codon ATG at position 30nt and a stop codon TAG at position 1127 nt using the DNAMAN program (Figure 1). None in-frame or out-of frame Met codon is found upstream of the presumptive ATG start codon, and the sequences near start codon match the Kozak rules well. The DNA sequence also contained a polyadenylation signal-like AATAAT at position 1180nt and a polyA tail. The full-length cDNA, named as *TaTMT*, contains a 1098 bp open reading frame (ORF) encoding a protein of 365 amino acid residues with isoelectric point (pI) 7.2 and calculated molecular weight of about 39,530 Da. This sequence has been sent to Genbank and the accession number is DQ139266.

Deduced protein analysis

A search of the Conserved Domain Database at NCBI confirmed that *TaTMT* belongs to the AdoMet_MTases super-family. All of the signature sequences including SAM binding domains were found in *TaTMT*. It is a chloroplast transit peptide predicted by TargetP algorithm, which is similar with its counterpart in *Arabidopsis*.

Sequence searches were performed using Blast with the putative full-length *TaTMT* protein sequence. Multiple full-length protein sequences, which showed high similarity to *TaTMT* in BLAST from various species, were used for multiple alignment and phylogenetic tree construction. Multiple sequence alignment shows that *TaTMT* protein shares high identity with the *γ-TMT* from rice (87%), maize (80%), tomato (75%), soybean (76%), potato (66%), *Arabidopsis* (70%), moss (60%), algae (48%) and *Synechocystis* (35%), respectively.

To investigate the evolutionary relationships among *γ-TMT* sequences, an unrooted phylogenetic tree was constructed using the Mega4.0 program (Figure 2). The results showed a clear evolution relationship. In general, γ-TMT evolved from *Synechocystis* , via algae and moss, to angiosperm. The *TaTMT* protein sequences of monocotyledons and dicots were clustered into two subunits, which indicated that they might evolve from a common ancestor gene. The phylogenetic analysis has also shown that three *γ-TMT* proteins from monocot plants (wheat, rice and maize) are more closely related to each other than to the proteins from other plants. In conclusion, these comparative studies strongly suggest that *TaTMT* is the homolog of *Arabidopsis γ-TMT* in wheat.

In Silico chromosome mapping of TaTMT

As of May 2009, 6596 wheat ESTs had been in the wheat

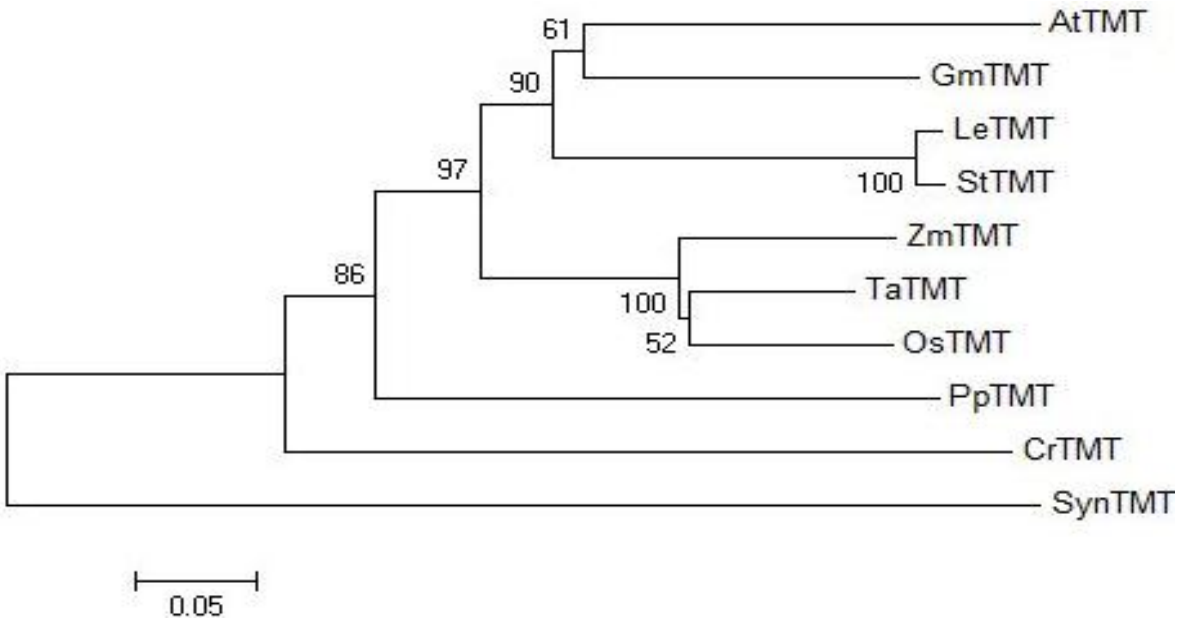

Figure 2. Phylogenetic analysis of plant γ-TMT genes. The tree was constructed using the neighbor-joining (NJ) method implemented in MEGA 4.0. The numbers beside the branches represent bootstrap values (>50%) based on 1000 replications. TaTMT(*Triticum aestivum*, AAZ67143), OsTMT(*Oryza sativa*, NP_001047844), ZmTMT(*Zea mays*, CAG25474), GmTMT(*Glycine max*, AAX63899), AtTMT(*Arabidopsis thaliana*, AAD02882), PpTMT(*Physcomitrella patens* subsp. *Patens*, XP_001758184), CrTMT(*Chlamydomonas reinhardtii*, XP_001694470), LeTMT(*Lycopersicon esculentum*, ABE41794), StTMT(*Solanum tuberosum*, ABE41795), SynTMT(*Synechocystis* sp. PCC 6803, NP_442492).

Chromosomes/chromosome bins using a set of wheat aneuploids and deletions stocks (Qi et al., 2004). An EST homologous sequence, BE607043, has 100% similarity with *TaTMT* cDNA (E-value 0.0) by searching database with BLASTN program. Based on sequence similarity and map position of the EST, the wheat *TaTMT* gene could be mapped in wheat 6AL, 6BL and 6DL chromosomes, which located in bin C-6AL4-0.55, 6BL5-0.40-1.00 and 6DL6-0.29-0.47, respectively (Table 1). As common wheat is a hexaploid species comprising of A, B and D partial homoeologous genomes, the gene we cloned in this study is one of the three genes but cannot be assigned to a defined genome and chromosome.

Table 1. Chromosomal locations of *TaTMT* gene.

Locus	Chromosome	Bin
BE607043		
NDS021BE607043-2	6DL	6DL6-0.29-0.47
NDS021BE607043-3	6BL	6BL5-0.40-1.00
NDS021BE607043-5	6AL	C-6AL4-0.55

Note: The EST sequence BE607043, which had been mapped in the 6th partial homoeologous chromosomes, shared 100% similarity with the TaTMT gene when searched by BLASTn algorithm program, indicating that they had the same chromosome location.

Digital expression profile of TaTMT

As of May 2009, there were 40,349 wheat Unigene clusters in NCBI database and expression profiles of the most Unigene clusters were generated based on counts of ESTs homologous to each Unigene. Since these ESTs transcript level. A search of UniGene database at NCBI showed that *TaTMT* transcript is detectable at all of the organs except for callus, cell culture and sheath (Table 2). Expression levels in these organs vary from 17 to 43 transcripts per million (TPM). Among the expression tissues, root has the lowest expression level with 17 TPM. These results of *in silico* expression indicated that were randomly selected for sequencing, their relative abundance in tissue may represent the corresponding *TaTMT* were preferentially expressed in photosynthetic organs. This is not a surprising result because *TaTMT* was located in chloroplast and tocopherols were synthesized in photosynthetic organs (Shintani and DellaPenna, 1998).

Gene expression under different tissues and high light stress

To validate the digital expression results, RT-PCR

Table 2. Expression profile of *TaTMT* gene in different tissues by *in silico* analysis.

Pool name	Transcripts per million (TPM)	Gene ESTs/ Total ESTs in pool
callus	0	0/10594
cell culture	0	0/10157
crown	73	1/13678
flower	31	2/64268
inflorescence	41	5/121088
leaf	34	2/57503
root	17	3/166795
seed	61	10/161877
sheath	0	0/1100
stem	42	4/93580

Note: This profile showed approximate gene expression patterns as inferred from EST counts and the cDNA library sources.

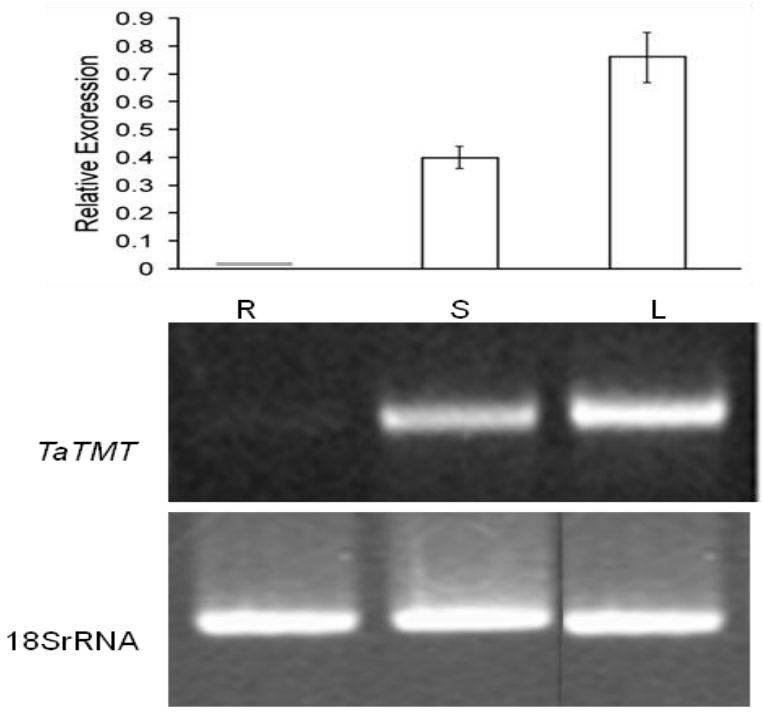

Figure 3. Expression analysis of *TaTMT* gene in different tissues (R, roots; S, stems; L, leaves).

analysis was performed to detect expression of *TaTMT* in roots, stems and leaves of common wheat. The results revealed that *TaTMT* was expressed in all tissues studied, but the levels of transcript abundance were obviously different (Figure 3). The *TaTMT* mRNA level was extensively high in leaves with relative expression value of 0.78, while it was lower in stems with relative expression value of 0.4. A very slight RT-PCR signal was detected in the RNA from roots, which relative expression value is nearly zero. These results were consistent with the above-mentioned *in silico* results, indicating that the UniGene database is a valuable and reliable resource for gene expression analysis.

To investigate the mechanisms of *TaTMT* transcription under stress condition, semiquantitative RT-PCR was used to detect *TaTMT* transcript levels at the different time points after exposure to high light. The results showed that the expression of the *TaTMT* was not regulated by high light stress (data not show). This is consistent with the findings in *Arabidopsis* (Shintani and DellaPenna, 1998). All of the four tocopherols as antioxidant have equal ability to relieve oxidative damage

caused by high light stress, whereas the function of *TaTMT* changes tocopherol types but not total volume.

In summary, a cDNA sequence encoding γ-tocopherol methyltransferase was isolated from common wheat and its expression pattern in various tissues was characterized by *in silico* and RT-PCR for the first time. The differential mRNA accumulation of *TaTMT* revealed that the expression of *TaTMT* was related to photosynthetic tissues and was not regulated to high light stress. In addition, *TaTMT* was found to be located on common wheat chromosome 6AL, 6BL and 6DL.

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