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Methodology

Molecular cloning and characterization of gene coding for γ-tocopherol methyltransferase from lettuce (*Lactuca sativa*)

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ABSTRACT. γ-tocopherol methyltransferase is an important rate-limiting enzyme involved in tocopherol biosynthesis. The full-length cDNA encoding γ-tocopherol methyltransferase (designated as *LsTMT*) was cloned from *Lactuca sativa* for the first time by rapid amplification of cDNA ends and characterized by means of quantitative RT-PCR. The full-length cDNA of *LsTMT* was 1131 bp, with an open reading frame of 897 bp encoding a γ-tocopherol methyltransferase protein of 298 amino acids, with a calculated molecular mass of 33.06 kDa and an isoelectric point of 5.86. Comparative analysis revealed that *LsTMT* has a close similarity with γ-TMTs from other plant species. Bioinformatic analysis indicated that *LsTMT* shares a common evolutionary origin based on sequence similarity and has the closest relationship to γ-TMT from the sunflower, *Helianthus annuus*. Based on quantitative RT-PCR analysis, we found that expression of *LsTMT* is induced and strengthened by oxidative stresses such as strong light and drought. The cloning and characterization of *LsTMT* will be helpful to further understanding its role

in the tocopherol biosynthesis pathway. We consider it to be a candidate gene for metabolic engineering of vitamin E in vegetable crops.

Key words: *Lactuca sativa*; γ-tocopherol methyltransferase; RACE; Quantitative RT-PCR

INTRODUCTION

Tocochromanols, known as vitamin E, are essential nutrients for human health. Tocochromanols are recognized as a group of compounds with amphipathic properties of eight derivatives and can be classified into two groups by the degree of saturation of their hydrophobic tails. The group characterized by a fully saturated tail belongs to tocopherols; while the tocotrienol group has an unsaturated tail. Both groups are composed of four members differing in the number and position of methyl groups on the aromatic ring, named α-, β-, γ-, and δ-tocopherol, respectively. α-tocopherol is a major isoform in most plant tissues except in seeds where γ-tocopherol is the dominant form.

The main function of tocopherol lies in its fatty acyl chain-breaking activity, which scavenges reactive oxygen species (ROS) resulting from photosynthesis, thus protecting polyunsaturated fatty acid chains (PUFAs) from lipid peroxidation. Increasing evidence suggests that in higher plants, vitamin E may play a protective role in cell membrane systems, thus maintaining the integrity and normal function of the photosynthetic apparatus (Havaux et al., 2005; Collin et al., 2008).

γ-tocopherol methyltransferase (γ-TMT) catalyzes the conversion of γ-tocopherol into α-tocopherol. So far, the *γ-TMT* gene has been cloned from *Arabidopsis thaliana*, *Brassica napus*, *Triticum aestivum*, *Helianthus annuus*, and many other plants. The overexpression of the γ-TMT gene (mostly from *A. thaliana*) has been reported in *Perilla frutescens*, *Lactuca sativa*, *Brassica juncea*, *Codonopsis lanceolata*, and in soybean seed (Van Eenennaam et al., 2003; Cho et al., 2005; Kim et al., 2005; Yusuf and Sarin, 2007; Lee et al., 2008; Seong et al., 2009), which demonstrated the increase of α-tocopherol. In this study, the gene encoding γ-tocopherol methyltransferase, *LsTMT*, was cloned from *L. sativa* for the first time. Its expression pattern is also analyzed by means of quantitative RT-PCR. The attainment of new genes involved in vitamin E biosynthesis could offer candidate genes for enhancing vitamin E levels of vegetables by transgenic engineering.

MATERIAL AND METHODS

Plant materials and isolation of RNA

Lactuca savita was grown at 25°C during the day/18°C at night in a chamber (16-h light/8-h dark cycle). Leaves of 3-month-old plants were used for total RNA isolation by using TIANGEN reagent following the manufacturer instruction (TIANGEN, China). After isolation, total RNA was used for cDNA cloning. The quality and concentration of RNA were measured by agarose gel electrophoresis and spectrophotometry before later steps.

Cloning of the full-length cDNA of *LsTMT*

Full-length cDNA of *LsTMT* was cloned by means of rapid amplification of cDNA ends

(RACE) according to the protocol of the SMART™RACE cDNA Amplification Kit (Clontech, USA). The core fragment of *LsTMT* was obtained using TMT-core1 (5'-TAGTTGATGTC/TGGGTGTGGT/C-3') and TMT-core2 (5'-TTTACATAATCAGCT/AGTAGAA/GCACC-3') as the PCR-amplification primers, designed based on two highly conserved amino acid blocks of γ-TMTs from other species. 5'-RACE-Ready cDNA of *LsTMT* was synthesized with 5'-CDS Primer A (5'-$(T)_{25}$ VN-3',V = A, G, or C) and SMARTII™A Oligonucleotide (5'-AAGCAGTGGTATCAACGCAGAGTACGCGGG-3') as primers, and total RNA isolated earlier as the template. Similarly, 3'-RACE-Ready cDNA of *LsG-TMT* was synthesized with 3'-CDS Primer A (5'-AAGCAGTGGTATCAACGCAGAGTAC$(T)_{30}$VN-3') as the primer, and total RNA isolated earlier as the template. Using the 3'-RACE cDNA as the template, primer *LsTMT3*'-GSP (GSP2: 5'-TACCTCCCGAAAAGTCCCTACGCCC-3') designed according to the sequence of the core fragment of *LsTMT* was applied as the forward primer and Universal Primer A Mix (UPM: 5'-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT-3'), which recognized the SMARTII A Oligonucleotides, as the reverse primer for the 3'-end cDNA amplification. The PCR amplification was performed as follows: 5 cycles (94°C for 30 s, 72°C for 2 min), 5 cycles (94°C for 30 s, 70°C for 30 s and 72°C for 3 min), 25 cycles (94°C for 30 s, 68°C for 30 s and 72°C for 2 min). Similarly, the 5'-RACE cDNA used as the template, primer *LsTMT5*'-GSP (GSP1: 5'-CTGGGCGTAGGGACTTTTCGGGAGG-3') that was also designed according to the sequence of the core fragment of *LsTMT* was applied as the forward primer and UPM as the reverse primer for the 5'-end cDNA amplification. The PCR amplification was performed under the same conditions as used for 5'-RACE above. The two amplified PCR products were purified and cloned into pMD18-T vector (TaKaRa, Japan) for sequencing. By comparing and aligning the sequences of 5'-RACE and 3'-RACE products, the full-length cDNA sequence of the *LsTMT* gene was obtained and its coding region was subsequently amplified using a pair of primers, TMT-ORF1 (5'-ATGGCGACTGCAGCGGATGAGC-3') and TMT-ORF2 (5'-TTATTCAGGCTTTTTACATG-3').

Bioinformatic analysis

Sequence alignments, open reading frame (ORF) translation and molecular mass calculation of predicted protein were carried out with Vector NTI Suite 8.0. BLAST (Basic Local Alignment Search Tool) was performed at the NCBI server (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi), whereas secondary structural analysis of predicted *LsTMT* was carried out on the website http://www.expasy.org. The phylogenetic analysis of *LsTMT* and γ-TMTs from other species was aligned with CLUSTAL W (a multiple sequence alignment program, version 1.82). MEGA (molecular evolutionary genetics analysis, version 2.1) was subsequently used to construct the phylogenetic tree by applying the neighbor-joining method.

Expression pattern analysis

SYBR Green I chimeric fluorescence quantitative real-time PCR was used to survey the expression pattern of *LsTMT* under high light and drought stresses. Total RNA was separately isolated from mature leaves of 8-week-old plants of lettuce, which had been treated with high light (light intensity: 1000 $\mu mol \cdot m^{-2} \cdot s^{-1}$) and drought (no irrigation) for 0, 3, 6, 9, 12, and 15 days (growing conditions: 16-h light/8-h dark cycle, at 25°C during the day/18°C at night).

Each of the 12 RNA samples was then reversely transcribed into cDNA with *TMT-RT1* (5'-GCTTGGTGTTCTACCGCTGATTATG-3') and *TMT-RT2* (5'-GCTCCTCTTATAGTCTT CCATCCAC-3') as primers, and cDNA as the template. PCR was performed with an approximately 200-bp long product. The PCR protocol was as follows: 1 cycle (95°C for 20 s) and 40 cycles (95°C for 15 s, 60°C for 15 s and 72°C for 25 s). And as a control, the ubiquitin gene was amplified following the above-mentioned PCR procedure. The PCR was repeated three times.

RESULTS

Cloning of the full-length cDNA of *LsTMT*

By using the above mentioned methods, the full-length cDNA of *LsTMT* was obtained. The full-length cDNA of *LsTMT* was 1131 bp long, including 5'- and 3'-untranslated regions, polyA tail, and a 897-bp ORF encoding a 298-amino-acid protein (Figure 1).

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GATTGTTGACGCAATACCACCACCACCACCACGAGGCAGCTTCTGCAGTCACTCAACACT
CAAGGAGTTATGAGTACGGTGGTTGCTGATGCAACAGTTCCTCCGATGACAATGGCGACT
                                                    M  A  T
GCAGCGGATGAGCAGCAGCAACAACAGCTAAAAAAGGAATAGCAGAATTCTACGATGAA
 A  A  D  E  Q  Q  Q  Q  L  K  K  G  I  A  E  F  Y  D  E
TCTTCGGGGATGTGGGAGAATATATGGGGAGAACACATGCATCACGGATTCTACGACACC
 S  S  G  M  W  E  N  I  W  G  E  H  M  H  H  G  F  Y  D  T
GATGCCGTCGTAGAACTCTCCGACCACCGCGCTGCTCAGATCCGTATGATCGAACAAAGC
 D  A  V  V  E  L  S  D  H  R  A  A  Q  I  R  M  I  E  Q  S
CTACTTTTCGCCTCTGTTCCTGATGATCCAGTAAAGAAGCCGAAAACCATAGTTGATGTT
 L  L  F  A  S  V  P  D  D  P  V  K  K  P  K  T  I  V  D  V
GGGTGTGGTATAGGAGGTAGCTCAAGGTACCTAGCAAGAAAATATGGAGCTGAATGCCAT
 G  C  G  I  G  G  S  S  R  Y  L  A  R  K  Y  G  A  E  C  H
GGCATCACCCTCAGCCCTGTTCAAGCTGAAAGGGCTCAAGCCCTAGCTGCTACCCAAGGA
 G  I  T  L  S  P  V  Q  A  E  R  A  Q  A  L  A  A  T  Q  G
TTAGCTGACAAGGTTTCATTTCAAGTTGCGGATGCTTTGAACCAGCCTTTTCCTGATGGA
 L  A  D  K  V  S  F  Q  V  A  D  A  L  N  Q  P  F  P  D  G
AAGTTTGACCTAGTTTGGTCAATGGAGAGTGGAGAACACATGCCTGACAAACTAAAGTTT
 K  F  D  L  V  W  S  M  E  S  G  E  H  M  P  D  K  L  K  F
GTTAGTGAGTTGGCTCGAGTGGCTGCTCCAGGAGCCACAATTATCATAGTCACATGGTGT
 V  S  E  L  A  R  V  A  A  P  G  A  T  I  I  I  V  T  W  C
CATAGGGACCTTTTACCTCCCGAAAAGTCCCTACGCCCAGAGGAAGAAAAAATCTTGAAC
 H  R  D  L  L  P  P  E  K  S  L  R  P  E  E  E  K  I  L  N
AAGATTTGTTCAGGATTTTTTCTTCCTGCTTGGTGTTCTACCGCTGATTATGTAAAATTA
 K  I  C  S  G  F  F  L  P  A  W  C  S  T  A  D  Y  V  K  L
CTCGAATCCATTTCCCTTCAGGACATCAAAGCAGAAGACTGGTCAGGAAATGTGGCACCA
 L  E  S  I  S  L  Q  D  I  K  A  E  D  W  S  G  N  V  A  P
TTTTGGCCAGCTGTGATAAAAACAGCCTTGTCTTGGAAGGGCATTACGTCATTACTAAGG
 F  W  P  A  V  I  K  T  A  L  S  W  K  G  I  T  S  L  L  R
AGTGGATGGAAGACTATAAGAGGAGCAATGGTAATGCCATCAATGATTGAAGGATTTAAG
 S  G  W  K  T  I  R  G  A  M  V  M  P  S  M  I  E  G  F  K
AAAGATGTAATAAAATTCTCCATCATTACATGTAAAAAGCCTGAATAAAAATAGGTGTGT
 K  D  V  I  K  F  S  I  I  T  C  K  K  P  E  -
AATGTACTTTGATTGTTGTACTTTCTTTCATAGACTGCTTCATTTTGGGTTCTTGTTTT
AATGTCTAATAACTCTTTTTTCAAAAAAAAAAAAAAAAAAAAAAAAAAAA
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Figure 1. The full-length cDNA and deduced amino acid sequence of *γ-TMT* from *Lactuca sativa*. The start codon (ATG) and the stop codon (TAA) are shaded.

Bioinformatic analysis

The deduced *LsTMT* had a calculated molecular mass of 33.06 kDa and a pI value of 5.86. The most frequent amino acid in deduced *LsTMT* was Ala (9.7% by frequency), followed by Leu, Lys and Ser (all 7.7%). Negatively charged residues (Asp+Glu) constituted 12.75% of the polypeptide, while positively charged residues (Arg+Lys) constituted 11.07% of the polypeptide. The protein was computed to have an instability index (II) of 44.64, which classified the protein as unstable (ProtParam, http://us.expasy.org/tools/protparam.html).

Protein-protein BLAST analysis showed that *LsTMT* had high homology to γ-TMTs from other plants (http://www.ncbi.nlm.nih.gov) (Figure 2), such as *H. annuus*, *Solanum tuberosum*, *T. aestivum*, *Gossypium hirsutum*, *P. frutescens*, *Elaeis oleifera*, *Zea mays*, *Hevea brasiliensis*, *Glycine max*, *B. napus*, *A. thaliana*, *B. oleracea*, with similarity of 90, 78, 76, 75, 76, 72, 73, 72, 77, 70, 72, and 71%, respectively.

The secondary structure of *LsTMT* was analyzed by SOPMA (Geourjon and Deléage, 1995) and the result showed that the putative *LsTMT* peptide contained 54.70% of α helix, 11.41% of extended strand, 3.69% of β turn, and 30.20% of random coil (Figure 3). The α helix constituted the majority of the secondary structure, and was the basic element of the N-terminal part, while the extended strand was the primary element of the C-terminal part.

The phylogenetic tree was generated using the present complete γ-TMT proteins deposited in NCBI. The tree indicated that *LsTMT* was the most homologous to γ-TMT of *H. annuus* (HaTMT) relative to γ-TMTs from other plants (Figure 4).

Expression profiles of *LsTMT* under high light and drought

Real-time RT-PCR was performed to investigate the *LsTMT* expression pattern under stresses like high light and drought. The results indicated that the expression of *LsTMT* was strengthened by the two stresses (Figure 5). Expression level of *LsTMT* gradually increased during 15 days of high light treatment (light intensity: 1000 $\mu mol \cdot m^{-2} \cdot s^{-1}$). During the session of drought (no irrigation) treatment, expression level of *LsTMT* peaked after 12 days of the treatment, and then declined remarkably after the peak.

DISCUSSION

γ-tocopherol methyltransferase is a crucial enzyme in tocopherol synthesis. In our study, we successfully isolated a γ-*TMT* gene from *L. sativa* for the first time. The deduced amino acid sequence of *LsTMT* has extensive similarity to its counterparts from other plants. All molecular analyses showed that *LsTMT* was quite similar in sequence to other γ-TMTs, especially to that from *H. annuus*, suggesting that *LsTMT* belongs to the γ-TMT family. The isolation and characterization of *LsTMT* will lead to profound research into its roles involved in tocopherol biosynthesis at the molecular level and provide useful candidate genes for enhancing the content of vitamin E in food by transgenic technique.

Up to now, many *γ-TMT* genes from different species have been found. A phylogenetic tree of γ-TMTs from different plant species was constructed. *LsTMT* was clustered with that from *H. annuus* (HaTMT), since they both belong to Compositae species (Figure 4).

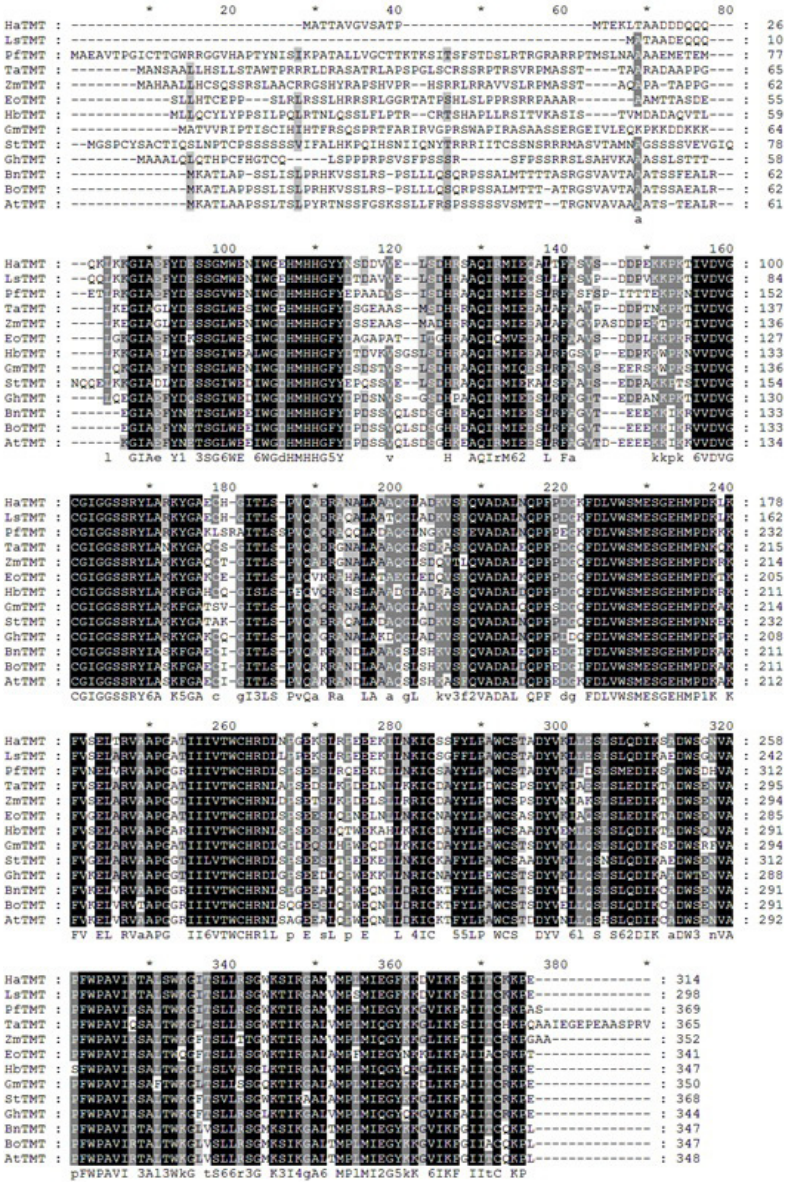

Figure 2. Multiple alignment of the deduced amino acid sequences of *LsTMT* and other γ-TMTs. Amino acid sequences were aligned as follows: LsTMT, HaTMT (*Helianthus annuu*s, ABB52798), StTMT (*Solanum tuberosum*, ABE41795), TaTMT (*Triticum aestivum*, CAI77219), GhTMT (*Gossypium hirsutum*, ABE41798), PfTMT (*Perilla frutescens*, AAL36933), EoTMT (*Elaeis oleifera*, ABS76142), ZmTMT (*Zea mays*, ACG34926), HbTMT (*Hevea brasiliensis*, BAH10645), GmTMT (*Glycine max*, AAX63899), BnTMT (*Brassica napus*, ACD03288), AtTMT (*Arabidopsis thaliana*, AAM64696), BoTMT (*Brassica oleracea*, AAO13806). Completely identical amino acids are indicated with capital letters against a black background. Less conserved amino acids are indicated with capital letters against dark-gray or relatively light-gray background. Non-conserved amino acids are indicated with black capital letters against a white background. Consensus sequence (the average sequence) is shown below the aligned sequences.

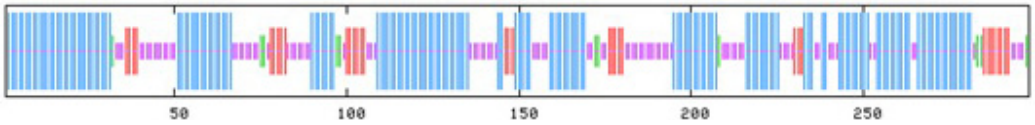

Figure 3. The secondary structure of *LsTMT*. Alpha helix, extended strand, beta turn, and random coil are indicated, respectively, with the longest, the second longest, the second shortest, and the shortest vertical lines.

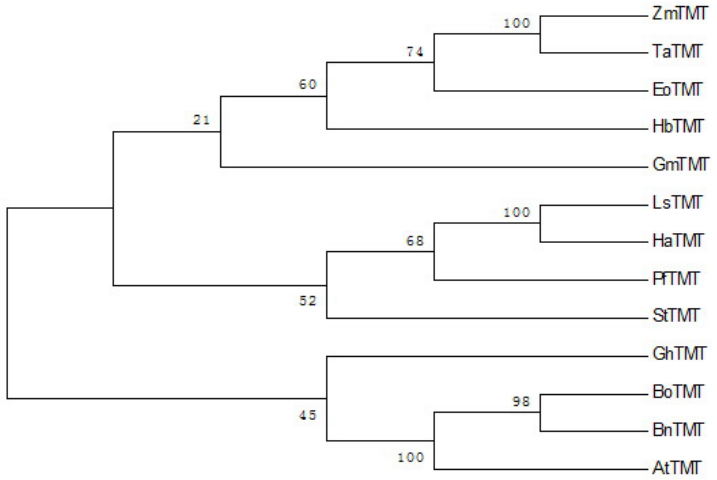

Figure 4. Neighbor-joining evolutionary tree of γ-TMTs. The tree was constructed with *LsTMT* and the sequences from NCBI as follows: HaTMT (*Helianthus annuu*s, ABB52798), StTMT (*Solanum tuberosum*, ABE41795), TaTMT (*Triticum aestivum*, CAI77219), GhTMT (*Gossypium hirsutum*, ABE41798), PfTMT (*Perilla frutescens*, AAL36933), EoTMT (*Elaeis oleifera*, ABS76142), ZmTMT (*Zea mays*, ACG34926), HbTMT (*Hevea brasiliensis*, BAH10645), GmTMT (*Glycine max*, AAX63899), BnTMT (*Brassica napus*, ACD03288), AtTMT (*Arabidopsis thaliana*, AAM64696), BoTMT (*Brassica oleracea*, AAO13806). The numbers on the branches represent bootstrap support for 1000 replicates.

ROS generated during photosynthesis and metabolism under oxidative stress can induce lipid peroxidation in plant cells and α-tocopherol content increases in response to a variety of abiotic stresses (Noctor, 2006) to prevent probable oxidative damage to lipid components by scavenging lipid peroxy radicals in the green parts of plants (Hare et al., 1998; Igamberdiev and Hill, 2004; Kiffin et al., 2006).

There is much evidence that tocopherol content correlates positively with tolerance to low temperature, high light, water deficit, and salt stress in different plants (Yamaguchi-Shinozaki and Shinozaki, 1994; Munne-Bosch et al., 1999; Guo et al., 2006). Induction of the expression of genes, including *γ-TMT*, in the tocopherol pathway promotes the accumulation of tocopherols under such stresses that may cause oxidative damage to a certain extent.

A previous study has shown that γ-TMT-silenced plants have strongly decreased salt tolerance (Abbasi et al., 2007). And overexpressing the *γ-TMT* gene greatly increased α-tocopherol level and enhanced tolerance to the stress induced by salt, heavy metal and osmoticum in *B. juncea* (Yusuf et al., 2010).

The quantitative RT-PCR result in our study demonstrates that the expression level of

the deduced *γ-TMT* gene from lettuce can be increased by high light and drought treatment on the transcriptional level (Figure 5), suggesting that more γ-tocopherol methyltransferases were generated for the synthesis of α-tocopherol, which may function as an antioxidant.

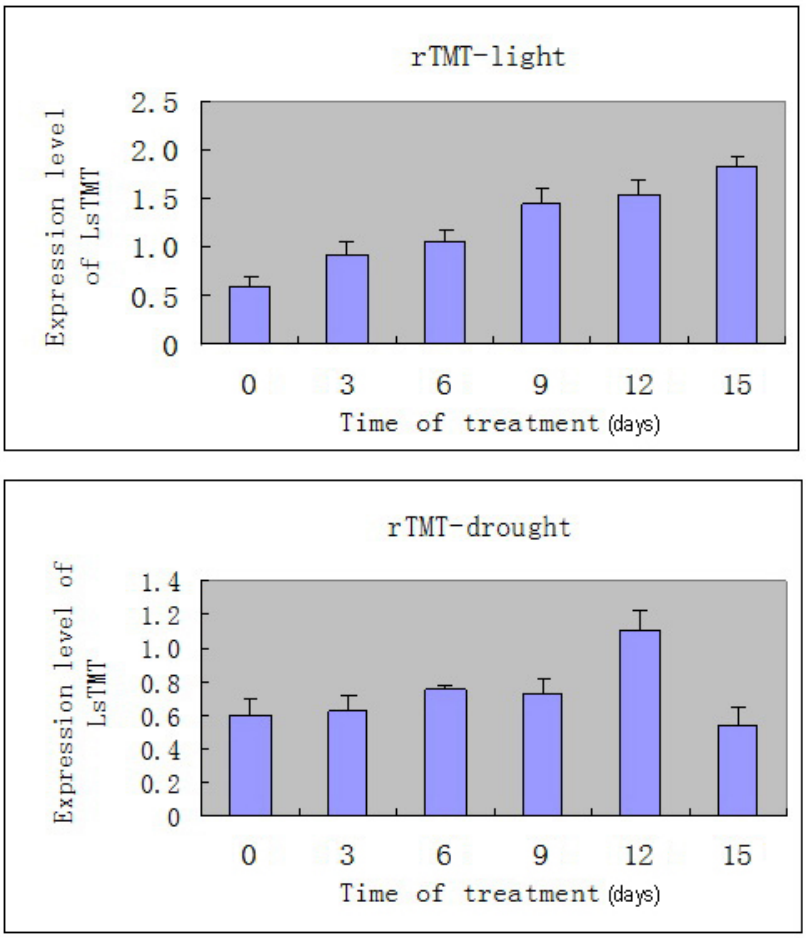

Figure 5. Real-time RT-PCR analysis of *LsTMT* expression. **A.** Expression profile of *LsTMT* under high light treatment for 15 days. **B.** Expression profile of *LsTMT* under drought treatment for 15 days. The RT-PCR experiment was repeated three times.

In the drought treatment, the expression level of *γ-TMT* declined markedly at the end, maybe as the plant was dying after an extended period of time without water, which severely affected its metabolism. In an earlier report, the expression level of several genes encoding ROS-scavenging enzymes was found to be upregulated in the *γ-TMT*-overexpressing deoduck plants (Seong et al., 2009). Our result, along with the above-mentioned reports, seems to indicate that γ-TMT can help to protect cells against oxidative damage caused by ROS under such stresses as water deficit, high light or salt, and so on. This study provides a profile of the expression pattern

of *LsTMT* under high light and drought stresses, which, together with the information about its sequence of deduced amino acids, revealed its possible function in plants' reaction against oxidative stress to some extent. However, there is still much to do to uncover the specific function of the isolated *LsTMT* gene in tocopherol metabolism by transgenic or gene knock-down techniques. And its regulatory mechanism on the molecular level also needs to be researched in the future.

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