GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

Molecular cloning and characterization of a tocopherol cyclase gene from *Lactuca sativa* (Asteraceae)

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Genet. Mol. Res. 10 (2): 693-702 (2011)
Received September 13, 2010
Accepted November 10, 2010
Published April 19, 2011
DOI 10.4238/vol10-2gmr1061

ABSTRACT. Tocopherol cyclase is a rate-limiting enzyme involved in tocopherol biosynthesis. The full-length cDNA encoding tocopherol cyclase (designated as *LsTC*) was cloned from lettuce (*Lactuca sativa*) for the first time by rapid amplification of cDNA ends (RACE) and characterized by means of quantitative RT-PCR. The full-length cDNA of *LsTC* was 1675 bp, with an open reading frame of 1521 bp, encoding a tocopherol cyclase protein of 506 amino acids, with a calculated molecular mass of 56.76 kD and an isoelectric point of 6.49. Comparative analysis revealed that *LsTC* has a close similarity with tocopherol cyclases from other plant species. Bioinformatic analysis indicated that *LsTC* shares a common evolutionary origin based on sequence and has the closest relationship to tocopherol cyclase from *Helianthus annuus*. Quantitative RT-PCR analysis suggested that expression of *LsTC* is induced and strengthened by oxidative stresses, such as strong light and drought. This cloning and characterization of *LsTC* will be helpful for further

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understanding of its role in the tocopherol biosynthesis pathway and provide a candidate gene for metabolic engineering of vitamin E.

Key words: *Lactuca sativa*; Tocopherol cyclase; RACE; Quantitative RT-PCR

INTRODUCTION

Tocochromanols, known as vitamin E, are essential nutrients in the daily diet of humans and animals. 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; in contrast, the group of tocotrienols has an unsaturated tail. Both groups are composed of four members differing in the number and position of methyl groups on the aromatic ring and called α-, β-, γ-, and δ-tocopherol, respectively.

The main function of tocopherols, especially α-tocopherol, which has been studied in more detail in humans and animals, lies in their fatty acyl chain-breaking activity, which could scavenge and quench various reactive oxygen species and protect polyunsaturated fatty acid chains from lipid peroxidation. Furthermore, it was found that they are involved in several signal transduction pathways and transcription regulation in organisms (Azzi et al., 2004). Increasing evidence suggests that in higher plants, vitamin E may play a role in protecting membrane systems in cells, thus maintaining the integrity and normal function of the photosynthetic apparatus (Havaux et al., 2005; Collin et al., 2008).

Tocopherol biosynthesis proceeds in plastids of photosynthetic organisms. The tocopherol biosynthesis pathway utilizes two compounds from different metabolic pathways as precursors, homogentisic acid (HGA) from the cytosolic shikimate metabolic pathway for head group synthesis and phytyl diphosphate (PDP) from the plastid methylerythritol phosphate pathway for tail synthesis of tocopherols or tocotrienols (Della-Penna and Pogson, 2006). There are at least five kinds of enzymes involved in the biosynthesis of tocopherols or tocotrienols, excluding the bypass pathway of phytyl tail synthesis and utilization. They are 4-hydroxyphenyl pyruvate dioxygenase (HPPD) for conversion of *p*-hydroxyphenylpyruvate (HPP) to HGA, homogentisate prenyl transferases (HPT) for prenylation of HGA to yield 2-methyl-6-phytylbenzoquinol (MPBQ) or 2-methyl-6-geranylgeranyl benzoquinol (MGGBQ), MPBQ methyltransferase for methylation of MPBQ and MGGBQ to yield 2,3-dimethyl-5-phytyl-1,4-benzoquinone (DMPBQ) and 2,3-dimethyl-5-geranylgeranyl benzoquinol (DMGGBQ), respectively, tocopherol cyclase (TC) for cyclization of MPBQ and DMPBQ to yield δ- and γ-tocopherol or for cyclization of MGGBQ and DMGGBQ to yield δ- and γ-tocotrienol, and γ-tocopherol methyltransferase for conversion of γ-tocopherol into α-tocopherol. So far, the genes coding for TC have been cloned and characterized from *Synechocystis* sp *PCC6803*, *Arabidopsis thaliana*, *Zea mays*, and many other plant species, which facilitates the research into the importance of this enzyme activity in affecting the rate of tocopherol synthesis (Provencher et al., 2001; Porfirova et al., 2002; Sattler et al., 2003; Kumar et al., 2005).

In this study, the gene encoding TC was cloned from *Lactuca sativa* for the first

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time, which is named *LsTC*. Its expression pattern was also analyzed by means of quantitative real-time-polymerase chain reaction (RT-PCR). The attainment of new genes involved in vitamin E biosynthesis could supply 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 by day/18°C by night in a chamber (16-h light/8-h dark cycle). Leaves of 3-month-old plants were used for total RNA isolation using the TIANGEN reagent following manufacturer instructions (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 *LsTC*

Full-length cDNA of *LsTC* 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 *LsTC* was obtained using TC-core1 (5'-GGAGTTGGAGCTCAGATCCTTGGT/GGC-3') and TC-core2 (5'-CCACTTTCTAGGG/AAAG/ACCTCCACCCC-3') as the PCR-amplification primers, designed based on two highly conserved amino acid blocks of TCs from other species. 5'-RACE-Ready cDNA of *LsTC* was synthesized with 5'-CDS Primer A (5'-$(T)_{25}$ VN-3', V = A, G, or C) and SMART II™A Oligonucleotide (5'-AAGCAGTGGTATCAACGCAGAGTACGCGGG-3') as primers, and total RNA isolated earlier as the template. Similarly, 3'-RACE-Ready cDNA of *LsTC* 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 template, primer *LsTC*3'-GSP (GSP2: GCCGGTTGGCTTGCTGCTTTTCCTG) designed according to the sequence of the core fragment of *LsTC* was used as the forward primer and Universal Primer A Mix (UPM: 5'-CTAATACGACTCACTATAGGG CAAGCAGTGGTATCAACGCAGAGT-3', which recognized the SMART II A Oligonucleotides, as the reverse primer for the 3'-end cDNA amplification. PCR amplification was performed as follows: 5 cycles (94°C for 30 s and 72°C for 2 min), 5 cycles (94°C for 30 s, 70°C for 30 s and 72°C for 3 min), and 25 cycles (94°C for 30 s, 68°C for 30 s and 72°C for 2 min). Similarly, 5'-RACE cDNA used as the template, primer *LsTC*5'-GSP (GSP1: 5'-ACAGGAAAAGCAGCAAGCCAACCGG-3'), which was also designed according to the sequence of the core fragment of *LsTC*, was used as the forward primer and Universal Primer A Mix (UPM) as the reverse primer for the 5'-end cDNA amplification. The PCR amplification was performed under the same condition 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 the 5'-RACE and 3'-RACE products, the full-length cDNA sequence of the *LsTC* gene was obtained and its coding region was subsequently amplified using a pair of primers TC-ORF1 (5'-ATGGACTCAAATCTGTATACATTGCTG-3') and TC-ORF2

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(5'-CTACAAACCAGGAGGTTTCAACAATG-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) and was performed at the NCBI server (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi), whereas secondary structural analysis of predicted *LsTC* was carried out on the website (http://www.expasy.org). The phylogenetic analysis of *LsTC* and TCs 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 determine expression pattern of *LsTC* under high light and drought stresses. Total RNA was separately isolated from mature leaves of 8-week-old lettuce plants, which had been treated respectively with high light (light intensity: 280 μmol m^{-2} s^{-1}) and drought (no irrigation) for 0, 3, 6, 9, 12, 15 days (growing condition: 16-h light/8-h dark and 25°C by day/18°C by night). Each of the 12 RNA samples was then reversely transcribed into cDNA. With *TC-RT1* (5'-GGAGCAAT TACAGTATGGACAACGG-3') and *TC-RT2* (5'-TTGCATAAGGTGTCTTTTCATCATC-3') as primers, and cDNA as the template, PCR was performed with a product of approximately 200 bp. 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). As a control, the ubiquitin gene was amplified following the above-mentioned PCR procedure. PCR was repeated three times.

RESULTS

Cloning of the full-length cDNA and genomic DNA of *LsTC*

By using the methods described above, the full-length cDNA of *LsTC* was obtained. The full-length cDNA of *LsTC* was 1675-bp long, including 5' and 3' untranslated regions, polyA tail, and a 1521-bp ORF encoding a 506-amino acid protein (Figure 1).

Bioinformatic analysis

The deduced *LsTC* had a calculated molecular mass of 56.76 kD and a pI value of 6.49. The most frequent amino acid in deduced *LsTC* was Gly (9.5% by frequency), followed by Glu (7.3%), Thr (7.1%), and Val and Leu (6.9%). Negatively charged residues (Asp+Glu) constituted 10.1% of the polypeptide, while positively charged residues (Arg+Lys) constituted 9.7% of the polypeptide. The protein was computed to have an instability index of 36.05, which classified the protein as stable (ProtParam, http://us.expasy.org/tools/protparam.html).

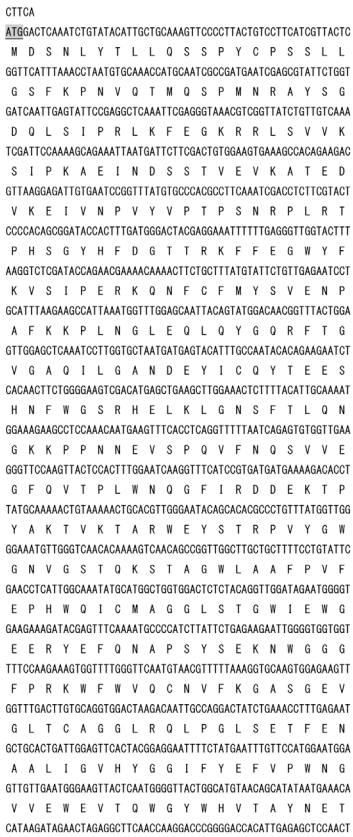

Figure 1. The full-length cDNA and deduced amino acid sequences of tocopherol cyclase from *Lactuca sativa*. The start codon (ATG) and the stop codon (TAG) are underlined and shaded.

ChloroP1.1 analysis (http://www.cbs.dtu.dk/services/ChloroP/) showed that *LsTC* contained an N-terminal chloroplast transit peptide of about 55 residues in length (data not shown), suggesting that this protein is located in the chloroplast.

Protein-protein BLAST analysis showed that *LsTC* had high homology to TCs from other plants (http://www.ncbi.nlm.nih.gov) (Figure 2), such as *Ricinus communis*, *Triticum aestivum*, *Phaseolus vulgaris*, *Arabidopsis thaliana*, *Solanum tuberosum*, *Eucalyptus gunni*, *Sesamum indicum*, *Hevea brasiliensis*, and *Helianthus annuu*s, with a similarity of 63, 58, 57, 61, 63, 65, 68, 66, and 76%, respectively.

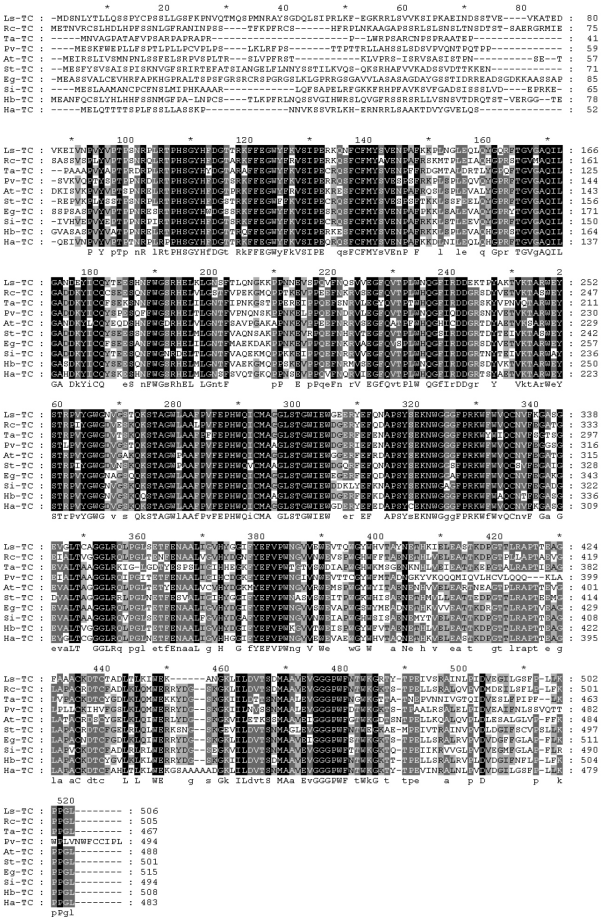

Figure 2. Multiple alignment of the deduced amino acid sequences of *LsTC* and other tocopherol cyclases (TCs). Amino acid sequences were aligned as follows: *LsTC* (*Lactuca sativa*), RcTC (*Ricinus communis*, EEF45889), TaTC (*Triticum aestivum*, ABE41800), PvTC (*Phaseolus vulgaris*, ACD50891), AtTC (*Arabidopsis thaliana*, NP_567906), StTC (*Solanum tuberosum*, AAT09809), EgTC (*Eucalyptus gunnii*, AAP97931), SiTC (*Sesamum indicum*, ABW98674), HbTC (*Hevea brasiliensis*, BAH10644), HaTC (*Helianthus annuus*, ABB52813). The completely identical amino acids were indicated with capital letters against black background. Less conserved amino acids were indicated with capital letters against dark-grey or relatively light-grey background. Non-conserved amino acids were indicated with black capital letters against white background. Consensus sequence (the average sequence) was shown below the sequences aligned.

The secondary structures of *LsTC* were analyzed by SOPMA (Geourjon and Deleage, 1995), and the result showed that the putative *LsTC* peptide consisted of 18.18% alpha helix, 24.11% extended strand, 6.52% beta turn, and 51.19% random coil (Figure 3). The random coil constituted the major part of the secondary structure, while alpha helix was the basic element of both N and C terminal parts.

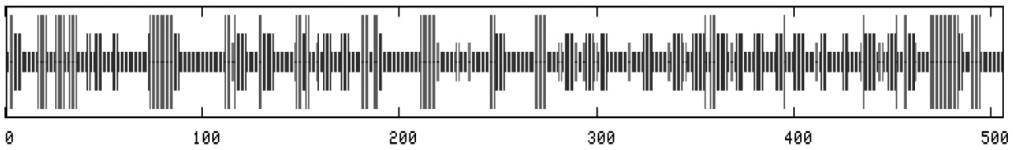

Figure 3. The secondary structure of *LsTC*. Alpha helix, extended strand, beta turn, and random coil are respectively indicated by the longest, the second longest, the second shortest, and the shortest vertical lines.

The phylogenetic tree was generated using the present complete TC proteins deposited in NCBI. The tree indicated that *LsTC* was the most homologous to TC of *Helianthus annuus* relative to TCs from other plants (Figure 4).

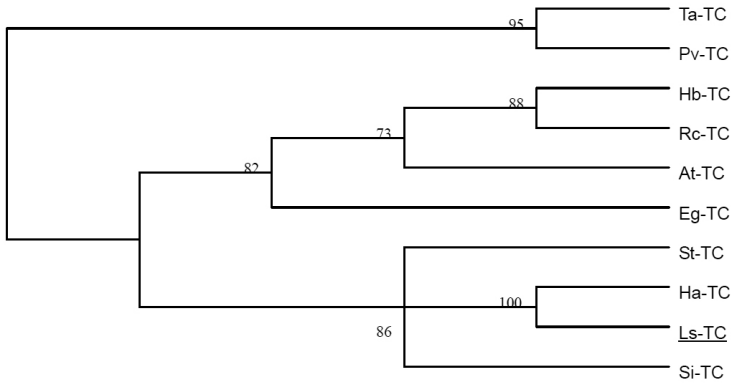

Figure 4. Neighbor-joining evolutionary tree of tocopherol cyclases (TCs). The tree was constructed with *LsTC* (*Lactuca sativa*, underlined) and the sequences from NCBI as follows: RcTC (*Ricinus communis*, EEF45889), TaTC (*Triticum aestivum*, ABE41800), PvTC (*Phaseolus vulgaris*, ACD50891), AtTC (*Arabidopsis thaliana*, NP_567906), StTC (*Solanum tuberosum*, AAT09809), EgTC (*Eucalyptus gunnii*, AAP97931), SiTC (*Sesamum indicum*, ABW98674), HbTC (*Hevea brasiliensis*, BAH10644), HaTC (*Helianthus annuus*, ABB52813). The numbers on the branches represent bootstrap support for 1000 replicates.

Expression profiles of *LsTC* under high light and drought

Real-time RT-PCR was performed to investigate the *LsTC* expression pattern under stresses, namely high light and drought. The results indicated that the expression of *LsTC* was induced and strengthened by the two stresses (Figure 5). Expression level of *LsTC* peaked after 6 days of high light treatment (light intensity: 280 μmol m^{-2} s^{-1}) and 9 days of drought (no irrigation) treatment, separately. *LsTC* expression level declined after the peak point under the two stresses.

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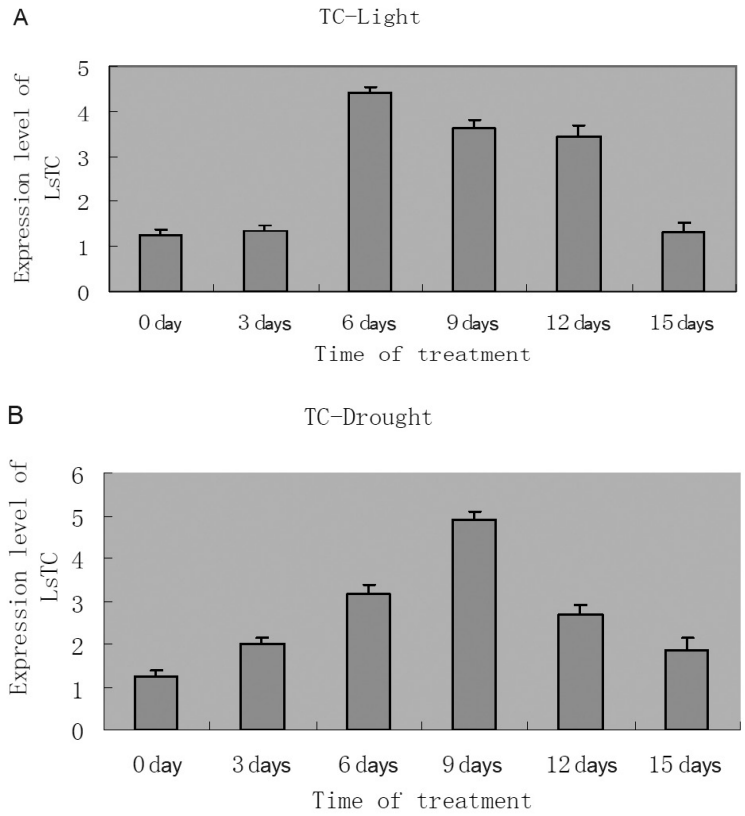

Figure 5. Real-time RT-PCR analysis of *LsTC* expression. **A.** Expression profile of *LsTC* under high-light treatment for 15 days. **B.** Expression profile of *LsTC* under drought treatment for 15 days. The RT-PCR experiment was repeated three times.

DISCUSSION

Tocopherol cyclase is a crucial enzyme in tocopherol synthesis. In our study, we successfully isolated a *TC* gene from *Lactuca sativa.* The deduced amino acid sequence of *LsTC* showed extensive similarity to the counterparts from other plants. All molecular analyses showed that *LsTC* was quite similar in sequence to other TCs, suggesting that *LsTC* belonged to the TC family. The isolation and characterization of *LsTC* will lead to profound understanding of its roles in tocopherol biosynthesis at the molecular level and provide an excellent candidate gene for enhancing the content of vitamin E in foods by transgenic techniques.

Up to now, a number of TC proteins from different species have been found. The discovery of a putative N-terminal chloroplast transit peptide in *LsTC* demonstrates that TC is transported to and functions in the chloroplast after its synthesis, which corresponds to previous reports (Vidi et al., 2006), since tocopherols are synthesized in the chloroplast of plants. A phylogenetic tree of TCs from different plant species was constructed. *LsTC* was clustered with TC from *Helianthus annuus*, since they both belonged to Compositae species.

An earlier study has shown that *TC* could endow plants with enhanced tolerance to drought stress, along with higher tocopherol content and lower lipid peroxidation levels (Liu et al., 2008). There is enough evidence that tocopherol content correlates positively with tolerance to low temperature, water deficit or 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 in the tocopherol pathway promotes the accumulation of tocopherols under stress to a certain extent, and these genes, including *TC*, may elicit different responses to different stresses (Kanwischer et al., 2005; Abbasi et al., 2007).

Quantitative RT-PCR results in this study demonstrated that the expression level of TC from lettuce was induced and increased by high-light and drought treatment at the transcriptional level (Figure 4), suggesting that more tocopherol cyclase was generated for the synthesis of vitamin E, which would act as an antioxidant to protect the membranes and proteins in the cell against the oxidative damage caused under such oxidative stresses as high light and drought. This study gives an expression profile of *LsTC* under oxidative stress, which, together with the information about its sequence of deduced amino acids, reveals its possible function in plants' response against oxidative stress to some extent. However, there is still much to do in the future to uncover its specific function in tocopherol metabolism and its regulatory mechanism at the molecular level.

ACKNOWLEDGMENTS

Research supported by the National Basic Research Program of China (973 Program, #2007CB108805) and Shanghai Leading Academic Discipline Project (#B209).

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